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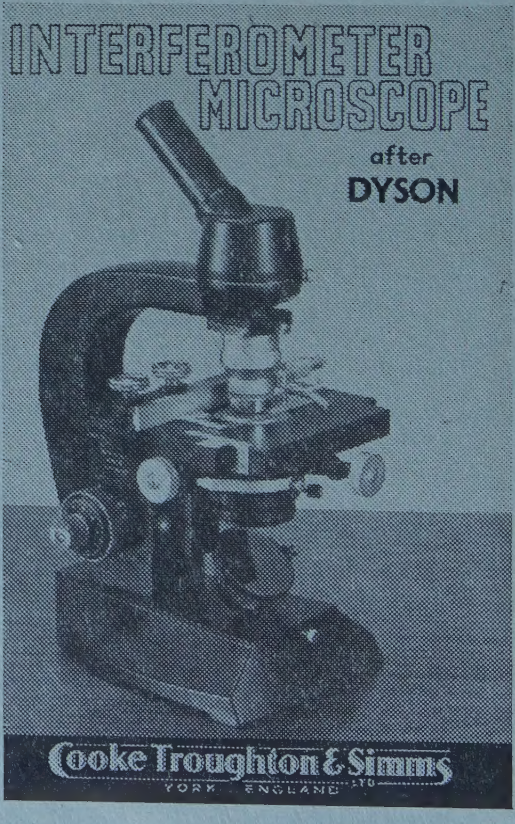


VOLUME 32, NO. 3 SEPTEMBER 1955

THE CORTICAL CHANGES ON FERTILIZATION OF THE SEA-URCHIN EGG

BY H. KACSER

Department of Animal Genetics, University of Edinburgh

(*Received* 28 *October* 1954)

The cortical changes which may be observed when the sea-urchin egg is fertilized have been the subject of a considerable number of investigations. The results have been discussed in relation to the problem of activation, and a number of conflicting views appear to be held at the moment. Three main lines of investigation give information on these processes. These are: (1) the kinetics of propagation in fertilized eggs, (2) artificial activation, and (3) the changes in mechanically constricted eggs. In what follows an analysis of the relevant evidence is presented. It will be suggested that a consistent picture of the nature of these processes can be given.

THE KINETICS OF THE DARK GROUND CHANGE

When the sea-urchin egg is fertilized, a change in the optical properties of the cortex spreads over the egg surface from the point of sperm attachment, this change taking about 20 sec. to pass over the egg surface at room temperature in *Psammechinus miliaris* (Rothschild & Swann, 1949). Rothschild (1949) examined the possibility that this cortical change might be due to a finite amount of a substance, derived directly or indirectly from the sperm head, diffusing round the cortex or through the egg cytoplasm. After comparing the rate at which the cortical change spreads over the egg surface with the appropriate theoretical diffusion curves, he concluded that the diffusion hypothesis could not be rejected, and that the intra-cytoplasmic route was more consistent with the experimental data than the cortical route. He also stated that the hypothesis of a simple diffusion mechanism might be oversimplified, or that the similarity between the theoretical and experimental curves might be fortuitous. There are several considerations which make the diffusion hypothesis difficult to sustain, and it will be shown that an autocatalytic mechanism initiated by the spermatozoon is more consistent with the data. Rothschild & Swann's raw data, which they were kind enough to allow me to re-examine, do not, however, enable a decision to be made between the intra-cytoplasmic and intracortical pathways.

ESTIMATION OF THE CHANGE

Rothschild & Swann's procedure was as follows. Photographs of the egg in optical section, taken at 0·5 sec. intervals, exposure 0·25 sec., show the spreading of a crescent (which appears dark in the negatives) from a certain point in the surface layer. The change reaches completion at a point approximately opposite to the beginning of the change. The rate of change was estimated from frame to frame on slightly enlarged prints by measuring the angle subtended at the centre by the arc.

Two possible sources of error arise from such a procedure. First, the use of prints introduces greater contrasts in optical densities and tends to 'smear out' finer details. Secondly, the decision as to where the 'centre' of the egg is will determine the angle which is recorded for a particular frame. An error in placing the centre will be negligible for small angles, but becomes important at angles in the neighbourhood of 180°. Thus, for example, an error of 7% in placing the centre can cause an angle of 178° to be read for a true angle of 160°. Since the cross-section of the egg is not perfectly circular this type of error is quite likely to occur. In order to eliminate both these difficulties, the following method was adopted in the re-examination of the material. The original film was used and the image of each frame was projected on to a carefully alined movable paper strip. The magnification resulted in an image of about 6 cm. diameter. The estimated arc was drawn and delimited for each frame. A graduated brass strip was bent into the exact shape of the arc and its length read off. This was then expressed as a fraction of the total circumference, thus avoiding the possible systematic error of the angular measurement.

While the use of prints resulted in the recording of definite arc lengths (or angles), the projection of the original film disclosed no definite end-points, but rather the tailing off of the optical change until it merged with the unchanged surface layer. A further uncertainty arose due to the structure of the egg material which resulted in considerable optical inhomogeneity. As a result each arc length could only be determined as lying between two limits: 'definitely changed' and 'definitely not changed'. With this procedure the variation between repeated readings (at different times) and between observers was considerable. It is therefore not justifiable to attach numerical significance to individual readings except as a qualitative indication of the rate of change.

A second significant difference between the two methods of measurement arose in determining the beginning of the change. The area where the measurable arc was first visible had shown, many frames previously, distinct changes of shape, structure and optical density. A slight flattening, a fuzziness and increase in grains indicated that something was happening.

A particular set of readings is shown in Fig. 1, together with the points recorded by Rothschild & Swann from print measurements of the same egg. It will be noted that the initial observations are indicated as points, although the observed changes often extended over considerable distances. Only when the optical change was a distinct and progressively increasing crescent were the limits recorded as lengths. In the middle regions the judgement was often extremely variable and often no data were recorded. Similarly, the end-point extended over several frames from 'probably complete' to 'definitely complete'.

In spite of the uncertainties involved, there are certain characteristics of the general shape which are relevant to an interpretation of the mechanism. These are:

the long induction period, the position of half completion and the inflexion of the average slope somewhere at this position. In Fig. 2 is given a composite plot from several sets of readings, indicating in a qualitative way the spread of the observations on one egg where the fertilization cone was visible.

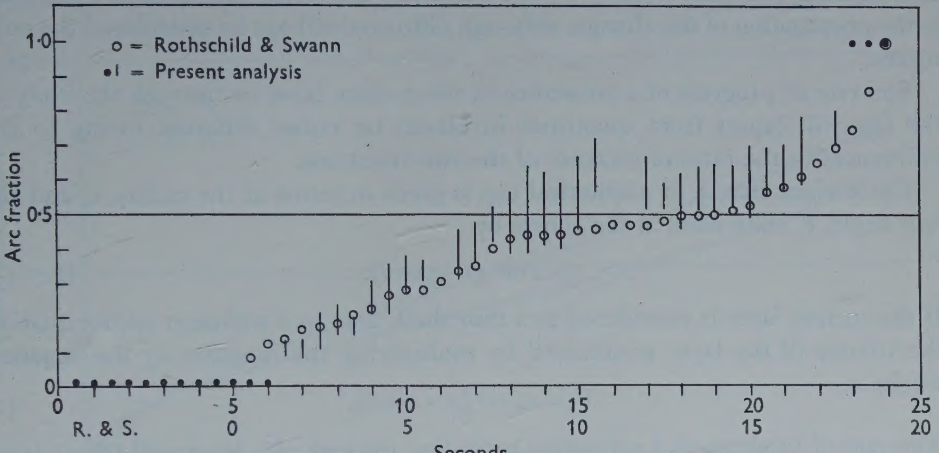


Fig. 1.

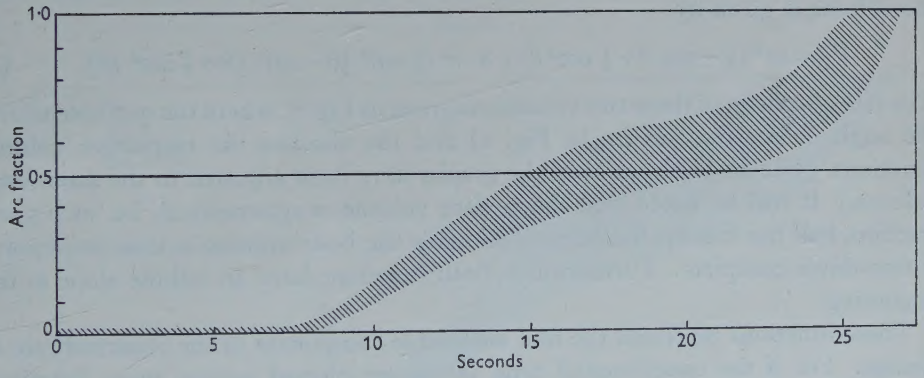


Fig. 2.

THE GEOMETRY OF THE MODEL

There are three main factors determining the observed rate of change: (1) the geometrical properties of the body, (2) the plane of observation relative to the change, and (3) the intrinsic rate of change.

The egg is very nearly a sphere. Calculations based on rotation ellipsoids show that the deviations are negligible compared with the experimental errors and therefore do not justify the more tedious analysis. The fact that the change spreads from a point over (or in) the surface may be due to a change in the surface layer only or to a change in the body of the egg which reacts with the differently constituted surface layer to produce the observed change. These two possibilities were dealt with by Rothschild and will also be considered here.

Arguments have been fairly convincingly advanced in favour of the spreading of some material substance rather than the propagation of an action potential. Consequently, in what follows it will be assumed that some substance is concerned in the propagation of the change, although diffusion will not be considered the only means.

The rate of progress of a substance in the surface layer or through the body of the egg will (apart from quantities involved) be rather different owing to the differences in the rates of increase of the two functions.

The surface area, s, of a spherical cap is given in terms of the radius, r, and the *half* angle, θ, subtended at the centre by

$$s = 2\pi r^2 (1 - \cos\theta). \tag{1}$$

If the surface layer is considered as a thin shell, then to a sufficient approximation the volume of the layer is obtained by multiplying the function by the constant height α.

$$V_s = \alpha 2\pi r^2 (1 - \cos\theta). \tag{2}$$

The rate of progress of a substance spreading through this layer will (apart from other variations) follow the rate of change of this volume. Similarly, if the substance is spreading through the body of the egg (with a spherical front of increasing radius), the volume is given by

$$V_B = \pi r^3 \left(\tfrac{2}{3} - \cos\theta + \tfrac{1}{3}\cos^3\theta\right) + 8\pi r^3 \left(\tfrac{2}{3}\sin^3 \tfrac{1}{2}\theta - \sin^4 \tfrac{1}{2}\theta + \tfrac{1}{3}\sin^6 \tfrac{1}{2}\theta\right). \tag{3}$$

The rate of change of these two volumes is given in Fig. 3, where the ordinate shows the angle or arc fraction (as in Fig. 1) and the abscissa the respective volume functions. (The absolute scales of the graphs have been adjusted to the same total volume.) It will be noted that the surface volume is symmetrical, i.e. at 0·5 arc fraction, half the volume has been filled while the body volume at that same point is two-thirds complete. Furthermore, both functions have an infinite slope at the beginning.

These functions represent the first analysable component of the observed rate of change. For if the experimental time values are plotted against these functions (Fig. 4) instead of angles or arc fractions, we obtain reduced rates of progress, i.e. plots which have eliminated the fact that the change takes place in or on a sphere. In other words, it is the true rate of spreading of the substance in one dimension or through a cylinder of unit cross-section. This transformation is only valid for 'equatorial' fertilization, when the plane of observation (optical section) is the same as the 'plane of spreading', i.e. the plane which passes through the starting-point and the centre of the sphere. When fertilization takes place above or below the plane of observation, the observed angle, θ, of the arc in that plane is not the true angle, ϕ, in the 'plane of spreading'. They are related to each other as follows, where A is the angle which the two planes make with each other

$$\cos\phi = \cos\theta \cos A. \tag{4}$$

It will be seen that for $\theta = 0$ (i.e. the beginning of the observation) the true angle already measures A. The difference between θ and ϕ will decrease until they become equal at $\theta = 90°$, i.e. the 'point of half completion', where the two planes cross. The observations will end ($\theta = 180°$) before the true change is completed, viz. at $\phi = (180 - A)°$.

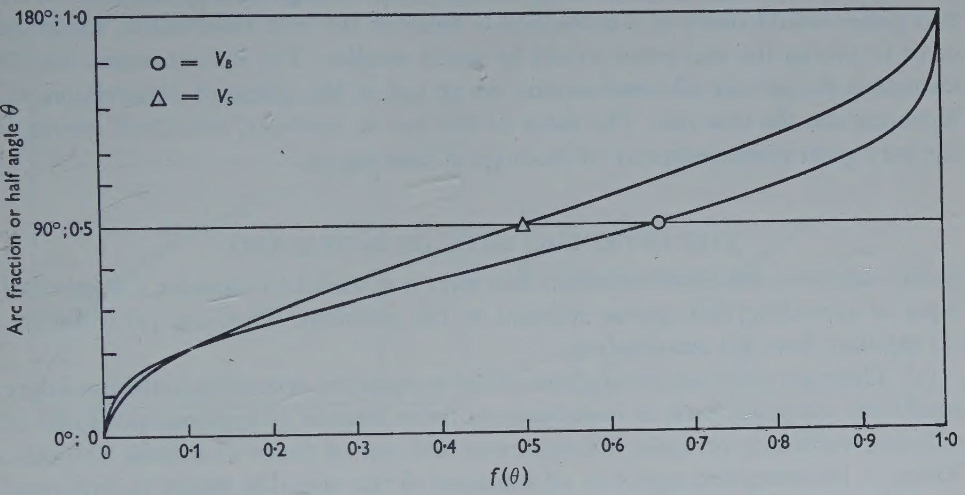


Fig. 3.

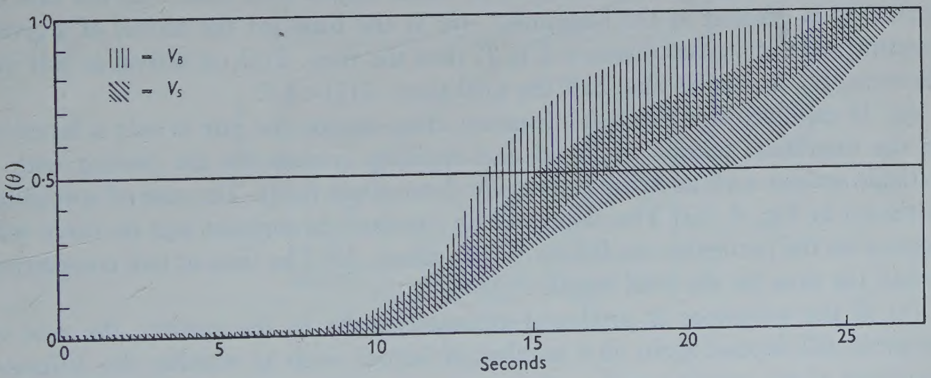


Fig. 4.

In terms of the time-angle plots this means that the period of observation is shortened both at the beginning and end. If the inclination of the two planes is ascertainable (by measurement of the distance of the fertilization cone to the plane of observation), a transformation of θ to ϕ is possible and equations (2) and (3) could then be applied to the ϕ values. In practice only those film records where sperm entry is near the equatorial plane of observation show the fertilization cone. But deviations of up to 15° for A will so be observed, since the depth of focus will record such a structure, although it is about 12μ above the plane, for an egg of 100μ

diameter. When measurements on such records are made on the assumption that the observed angle is the true angle, this possible source of error must be taken into account.

Only when the intrinsic rate is uniform will the shortening due to angle A be symmetrical. If, as will be shown later, the intrinsic rate is small at the beginning and increases towards the end of the change, a small angular displacement of the two planes would result in a considerable delay of the first observation, while the error in timing the end-point would be much smaller. The instantaneous rate of change at $\theta = 90°$ for all fertilizations, in or out of the plane of observation, is, however, also the true rate. The value of this fact is, however, restricted, owing to the very great indeterminancy of readings in that region.

THE INTRINSIC RATE OF SPREADING

Before analysing the observations in this way, it is useful to consider a number of types of spreading that appear relevant to the problem. They are (1) diffusion, (2) capillary flow, (3) autocatalysis.

(1) There are many solutions of the diffusion equation, according to the boundary conditions assumed, such as instantaneous finite sources or continuous supply of diffusing substance or again diffusion into and out of finite or infinite volumes. There is, however, one aspect of all solutions of the equation which is important for our consideration. This is given by Fig. 5. This shows the type of curve which the progress of a given concentration of solute shows with time. (*a*) the rate of spreading is greatest at the beginning. (*b*) If the time for the arrival of a given concentration at a given distance L is T, then the time, $T(\frac{1}{2})$, of arrival at half the distance, $\frac{1}{2}L$, is smaller than half the total time: $T(\frac{1}{2}) < \frac{1}{2}T$.

(2) In capillary flow through a constant cross-section the rate is only a function of the interfacial tension of liquid and capillary (unless we are dealing with a variable system such as hydrostatistically determined flow). The rate of spreading is shown in Fig. 6. (*a*) The slope will be constant throughout and its value will depend on the particular conditions of the system. (*b*) The time of half completion is half the time for the total length $T(\frac{1}{2}) = \frac{1}{2}T$.

(3) If the substance is produced autocatalytically in the system, the rate of progress will depend again on a number of factors, such as whether the diffusion constants of the reacting and reacted species are the same or different. Furthermore, the relation of the concentration dependence of diffusion and autocatalysis will be important. All such cases, however, will show the general behaviour given in Fig. 7. (*a*) The rate is smallest at the beginning. (b_1) The time of half completion is greater than half the time for the total length $T(\frac{1}{2}) > \frac{1}{2}T$; or, ($b_2$). If the initial amount of catalyst is such that the system is nearing equilibrium at L, $T'(\frac{1}{2})$ may be smaller than $\frac{1}{2}T'$, but in that case the inflexion point will be between $t = 0$ and $t = T'(\frac{1}{2})$.

The precise form of the equation will be rather complex, being a diffusion equation with autocatalysis as boundary conditions, the possibility of a shape factor entering as well. But the general characteristics will be those described.

On comparing these three models with the experimental evidence in the 'reduced' plots of Fig. 4, it is clear that diffusion cannot be the relevant process. The slope is not maximal at the beginning and $T(\frac{1}{2}) \nless \frac{1}{2}T$. This is also evident from the curves given by Rothschild (1949, figs. 1 and 2). All the theoretical curves have $T(\frac{1}{2}) < \frac{1}{2}T$ and all the experimental curves $T(\frac{1}{2}) > \frac{1}{2}T$. This is a far more sensitive test of agreement than the general shape which, from the evidence of Fig. 3, will always be sigmoid.

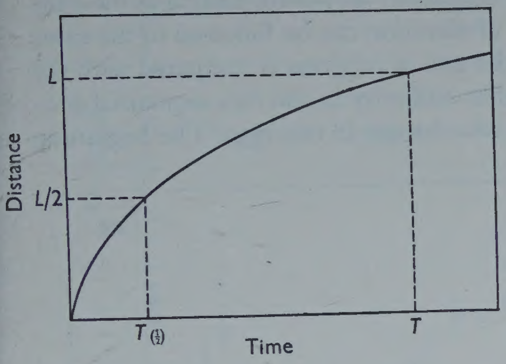


Fig. 5.

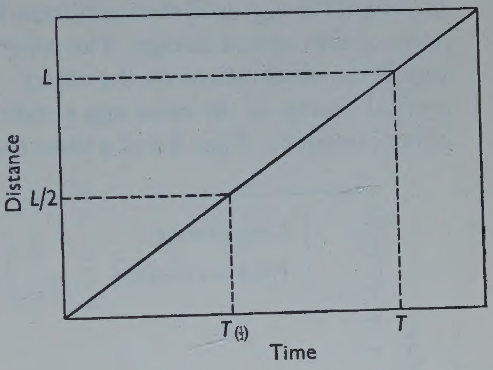


Fig. 6.

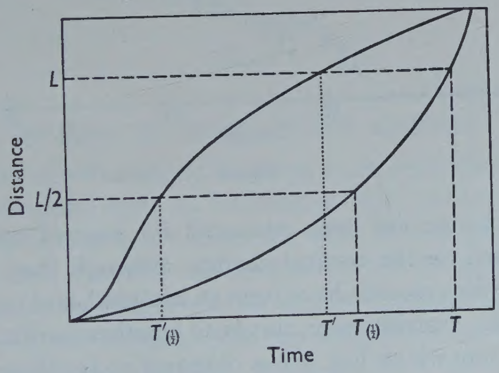


Fig. 7.

Capillary flow (an unlikely mechanism in any case) does not agree in its slope and time sequence either. The rate of progress of the *observed* 'unreduced' change would correspond to the curves in Fig. 3 as these represent also the filling of the respective volumes by a constant supply. It is clear that either the initial slope or the half-time or both do not correspond to the experimental curve (Fig. 2).

Some process involving autocatalysis, on the other hand, appears to fit the experimental data best. The uncertainties in the data make it unprofitable to attempt the evaluation of an equation. For the same reason it is not possible to decide between the alternatives of surface and body spreading.

THE FERTILIZATION MEMBRANE

There is another phenomenon which seems to stand in a relatively simple relationship to the cortical change. This is the elevation of the fertilization membrane which takes place shortly after the cortical change. About the time of completion of this change, the membrane begins to separate from the egg at the place where the cortical change started. It is visible as a crescent standing out from the body of the egg attached at two more or less defined positions. These positions move along the surface of the egg until the membrane has completely separated, ending at the end-point of the cortical change. The progress of elevation can be followed in the same way as the observations on the cortex. If the rate of progress is compared with the cortical change of the same egg a remarkable similarity in the two sequential processes is evident. Figs. 8 and 9 show these two changes in two eggs. The beginning of the membrane change has been advanced for ease of comparison so that it appears superimposed on the cortical change, although they occur one after the other. A number of film records have been so analysed, and in each case the agreement is unmistakable. Furthermore, this is so whether fertilization was equatorial or not. In the egg from which Fig. 9 was obtained no fertilization cone was visible and the total time was, as expected, significantly shorter than for the egg of Fig. 8. Yet the curve for the lifting of the membrane runs parallel with that for the cortical change.

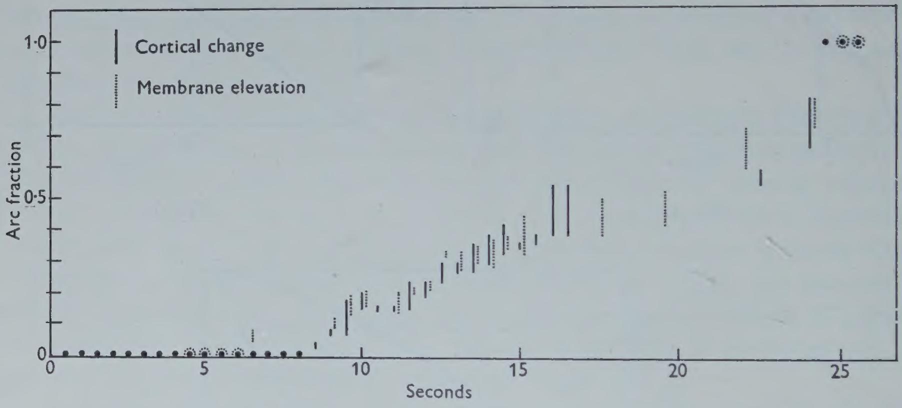


Fig. 8.

An interpretation of this correlation can be given as follows. If the observed cortical change is a precursor of a series of reactions resulting in the elevation of the membrane, then the cortical reaction must be the rate-determining step, i.e. it is slower than all other processes.

Alternatively, the lifting of the membrane may be the physical consequence of the completion of the cortical reaction. The observed optical change may be the first signs of a single condensation or polymerization process which results in the production of material which unites with the vitelline membrane.

In any case, the close correlation of the two observable phenomena strengthens the reality of the time sequence, and makes it possible to measure the 'same reaction' by two observationally independent methods.

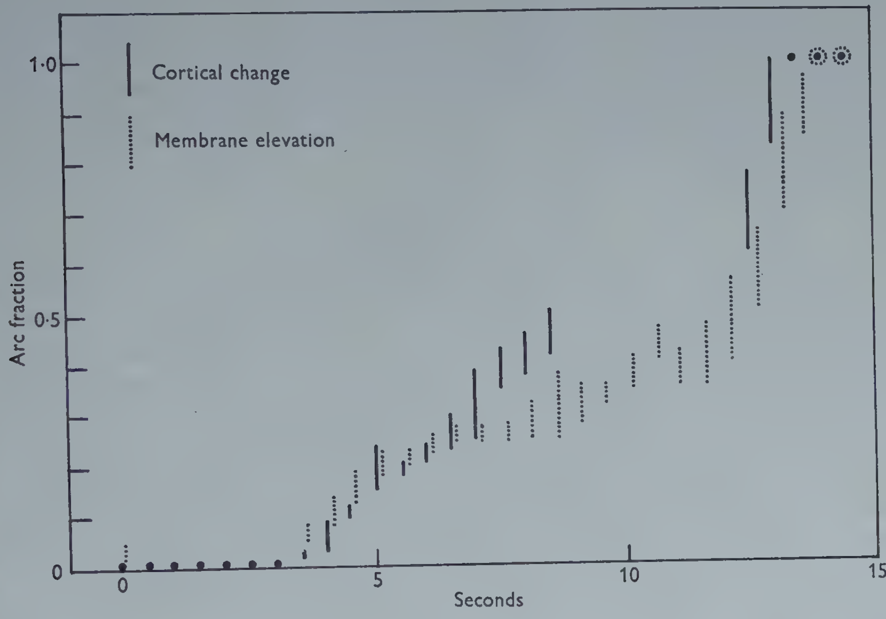


Fig. 9.

EVIDENCE FROM OTHER SPECIES

We shall now consider whether any of the other work which has been carried out on the reaction of echinoderm eggs to fertilization and particularly that involving artificial activation, provides data compatible with an autocatalytic mechanism or will enable us to decide between the alternatives of the cortical or cytoplasmic routes for its transmission. There are no strictly comparable reports for *Psammechinus*. However, Moser (1939*a*), studying *Arbacia punctulata*, observed a wave of roughening spreading over the surface of the egg from the point of sperm entry, followed, shortly after the completion of the change, by the elevation of the membrane starting from the same point. The roughening appears to be due to the breakdown of granules lying immediately beneath the cortex. The structural nature of the change is thus different from that observed by Rothschild & Swann in *Psammechinus*, where the increase in light scattering is probably due to the formation of a more granular structure. However, while the optical concomitants of the change will depend on the state of aggregation of the layer, which may be different in the two genera, the underlying chemical basis of the change may well be the same. In view of the identity of the response sequence of the two processes in the two genera, we suggest that we are concerned with the same intrinsic phenomena. It is worth pointing out that Moser's theory that membrane formation depends on a vacuolization consequent on the breakdown of granules is difficult to accept unless one makes the assumption that the membrane is produced in two different ways in *Arbacia* and *Psammechinus*. There are no quantitative data for the kinetics of the change, but Moser gives an average value of 9·9 sec. from fifty-seven measurements. For reasons discussed by Rothschild & Swann in their paper, the longest and not the average time would be nearer the true response time. Here again, however, differences in temperature, size, viscosity and state of aggregation from one form to another could result in absolute differences without implying a difference in the intrinsic nature of the response.

ARTIFICIAL ACTIVATION

Moser (1939*b*, 1940) studied a considerable number of agents (saponin, toluol, urea, thiourea, glycerine, sucrose, mechanical puncture, ultraviolet and direct current) which can act as artificial activators and produce the cortical response followed by the elevation of the membrane. On the autocatalytic hypothesis one might expect initiation to be caused by the introduction of some of the catalyst. These observations show that the initiation of the cortical response cannot be entirely dependent on the spermatozoon bringing in a quantity of catalyst. One must suppose that the action of the spermatozoon or of the artificial activators either enables a catalyst to enter from the sea water, or initiates the release of a catalyst already contained in an inactive form in the egg. The evidence that the response is inhibited by the removal of calcium ions from the water and, according to Moser (1939*b*), 'presumably from the cortex', might suggest that the calcium ions are the actual initiators, but it is equally possible to suppose that they are concerned not with the initiation but with the capacity to carry out the response. We shall return to this aspect later. There is then nothing in these observations incompatible with the suggestion that a catalytic agent is released from within the egg itself. In fact, the occurrence of artificial activation might be expected on our hypothesis since the mechanism does not require the intervention of any *specific* agent.

It is also not unexpected to find that when an activating agent (urea solution) is allowed to flow from one side into the drop containing the egg, the cortical change, followed by the membrane, proceeds from this side in a wave-like manner, while if the eggs are placed in a solution (e.g. saponin) so that there is no preferential direction of exposure, the response occurs more or less simultaneously over the whole surface. From our point of view a more important observation is that of Kitching & Moser (1940), who found that absence of oxygen does not prevent or even slow down, the cortical change or the membrane elevation, although it inhibits cleavage. Furthermore, the time relations of the cortical change and membrane elevation, initiated by saponin, urea, sucrose and puncture, were the same with or without oxygen. This rather strongly suggests the autocatalytic break-down of a metastable system which, once it has started, would be self-perpetuating and would be independent of the free energy supply of the respiratory system.

THE ROLE OF THE CYTOPLASM

When *Arbacia* eggs are strongly centrifuged the cortical layer of granules will remain in position even when the inner cytoplasm is stratified. Such eggs do not lose the ability to exhibit the cortical reaction and membrane elevation, but the time-relations between these two processes are frequently altered. Thus Moser (1939*a*) has shown that following fertilization at the centrifugal pole, membrane elevation begins when the cortical reaction has reached half completion, although in uncentrifuged eggs (as in *Psammechinus*) this reaction is complete before the elevation begins. He reports (1939*b*), precisely the same relations when initiation is effected by saponin applied at the centrifugal end. He further found that while centrifuged eggs can apparently be stimulated by spermatozoa at any point on the surface, saponin can initiate the response only at the centrifugal and puncture only at the centripetal end. These facts suggest that different parts of the egg may have different thresholds or quantitatively different initiation responses at least with respect to certain activating agents. At any rate the evidence clearly indicates that the condition of the internal cytoplasm has a considerable effect on the progress of the cortical reaction. In view of this it would be difficult to suppose that the processes which bring about the spreading of the cortical reaction involve that layer only. It might still be, however, that the change is transmitted primarily within that layer but involves the mobilization into it of catalyst released at the place of initiation from the underlying cytoplasm, or of substances necessary for propagation, this mobilization being influenced by the stratification of the cytoplasm in centrifuged eggs.

Motomura (1934, 1941) has described a cortical layer in *Strongylocentrotus pulcherrimus*, which he considers comparable to Moser's granular layer. He describes the discharge of the granules from the cortical zone on to the membrane following fertilization, or activation with butyric acid or urea. This discharge is a very slow process, taking as long as 10 min. It seems to be concerned mainly with the hardening of the fertilization membrane and not with its initial elevation. He does not describe any cortical reaction which preceded the elevation of the membrane, and it is possible that in this form the reaction, if it occurs, is unaccompanied by any optical change. The process described by Motomura is therefore not comparable to the reactions we have been discussing, and does not provide any reason to doubt the hypothesis which has been advanced.

CONSTRICTED EGGS

In a recent publication (Allen, 1954) experiments on fertilization of *Psammechinus miliaris* eggs, drawn into capillaries of various diameters, are reported. It was possible to follow the cortical granule transformation, subsequent to fertilization at one end, along the cylindrical portion of the egg. In view of the constriction in the tube the elevation of the membrane did not take place (except at the end or ends), although it could be shown in some cases that the membrane had separated by the possibility of sliding the eggs back and forth in the membrane.

As in the present report, the wave of propagation had no sharp front but showed a region (20–30μ) in which the granules were breaking down at random. They were smaller and became less plentiful towards the portion already changed. In dark ground a yellow colour is replaced in a wave-like manner by a brilliant white. Here, too, the intermediate region showed patches of yellow.

In twenty-nine cases the cortical change proceeded to completion to the far end of the egg, but in fifty-six cases (the figure 63 is mentioned in the text) the change apparently 'died out at some point on the cylindrical surface'. The rate of change of cortical transformation was measured in ten of the eggs by noting the time that single granules broke down first 'at known distances from the point of sperm entrance'. Zero time was assumed as the time when the surface of the spherical portion of the front end began to roughen, 'evidently caused by the breakdown of cortical granules'. Only three representative curves are given and these are reproduced in Fig. 10. The ordinate does not show the observed distances but area of response covered (calculated, from the distances and the value of 97μ for the diameter of the spherical egg, on somewhat inaccurate assumptions). This representation is similar to that given by the V_s curve in Fig. 4 although it would have been more useful for comparison purposes to express these as fractions of total surface instead of absolute μ^2.

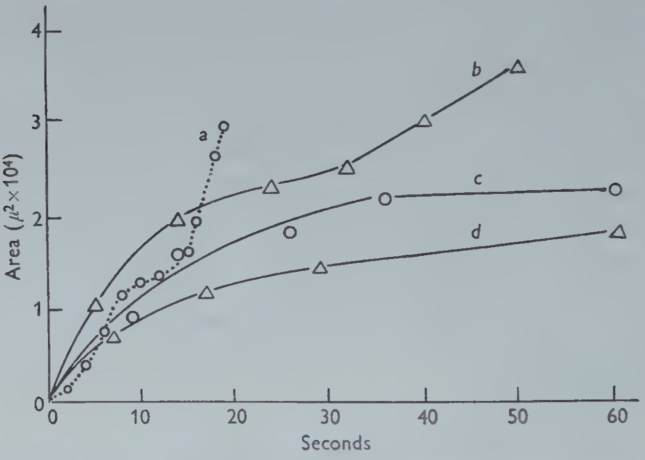


Fig. 10. The rate of the fertilization impulse: (*a*) curve for the rate of the dark-field colour change impulse, recalculated from data of Rothschild and Swann (1949); (*b*) an ATP pretreated egg (length 188μ) in which the impulse passed over the entire surface; (*c*) a dispermic egg (length 423μ); and (*d*) a monospermic control egg (length 396μ). (Curves *b*, *c* and *d* were constructed from observations on the breakdown of individual cortical granules at different distances from the point of sperm entry.) (From Allen, 1954.)

The main points to note are the greatly extended time and the shape of the curves. Curves *c* and *d* clearly correspond to diffusion curves (cf. Fig. 5). In the absence of the present analysis this might have been considered powerful support for Rothschild's hypothesis. Curiously enough Allen considers these as evidence against it, asking why, since there has been no change in either cortical or cytoplasmic *volumes*, these 'should still not be filled by the amount of diffusing substance introduced by one, or even two, spermatozoans'.

The answer is as follows. The evidence presented does *not* show that the volume is not being filled but that the *rate* at which it is being filled decreases with the increase in distance. Now the rate is, of course, a function of the geometry of the egg. Allen considers 'stretching, straightening of the egg surface and contact with the glass: three inseparable variables' as the only factors that have been changed, while of course the diffusion *paths*, both cortical and cytoplasmic, have been increased and the cross-sectional areas decreased. The time scale of diffusion will therefore be 'stretched' considerably.

Allen offers an explanation of the results by postulating 'evenly spaced *units* of some sort' in the cortex which can exist in 'non-activated or activated forms'. He then postulates that the spermatozoon initiates a 'chain reaction' among the units, a suggestion already hypothecated by Runnström & Kriszat (1952). (It is clear that the term 'chain reaction' is not used in its accepted chemical sense.) Both these authors and Allen cite, in support of the hypothesis, the observed acceleration of the wave when it passes over the end portion of decreasing area. It has already been pointed out (Fig. 3) and is also evident from Rothschild's theoretical curves that, for geometrical reasons, *any* process travelling over that portion will show acceleration.

To account for the decreasing slope of the curves he postulates 'that the number of units activated decreases for each successive unit of time'. On the other hand, to account for the cases of 'complete fertilization' (over 35 % of the control eggs), when the slope increases, he postulates the opposite, viz. 'the successful conversion of an increasing proportion of units'. With this somewhat flexible hypothesis, the difference in behaviour between elongated and normal eggs is explained by reference to a 'disruption' of 'the structure in which the units we have postulated play an integral part' or 'due to the fact that the postulated units become farther apart' because of stretching.

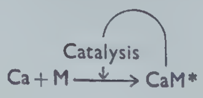
The disruption explanation is made unlikely by the fact of 'continued complete fertilizability of the entire unfertilized part' and by the fact that the return of stretched eggs to a solution 'removes the inhibition of membrane formation at the sides in contact with the capillary and, as a rule, at the hind end as well' (Hörstadius & Runnström, 1953).

The stretching explanation is contradicted by the observation of Runnström & Kriszat (1952) on eggs which became attached to the bottom of a glass dish. The propagation of the impulse was inhibited in that part of the surface which was in contact with glass. This portion is not stretched, if anything, it is compressed. As in the capillary experiments, in a number of cases when such 'partially fertilized' eggs could be detached without injury, normal membrane formation could be induced in that portion.

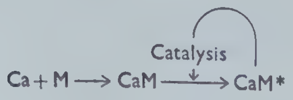
It is therefore clear that, under the prevailing experimental conditions, the evidence presented shows the propagation of the response to obey not an autocatalytic mechanism but strongly suggests a diffusion process. This apparent contradiction between Allen's results and those of Rothschild & Swann can be resolved if it is remembered that calcium ions are necessary for the response to take place. Now, apart from the geometrical parameters already discussed, eggs inside capillaries as well as the attached eggs differ from the spherical eggs in solution by having part of their surface not in contact with calcium containing water. The necessary calcium can only be brought to the site of reaction either by diffusion through the egg or through the space between egg and glass.

Runnström & Kriszat report that their jelly-free eggs adhered strongly to the glass which would provide a very good seal. The complete failure of the response in the adhering portion even after prolonged times (up to 120 min.) shows that the calcium does not diffuse through the egg or cortex. Adhesion could be prevented by the addition of 0·1–0·2% serum albumin, a procedure adopted throughout Allen's experiments. Under these conditions diffusion can take place through the thin annular space between the egg and the capillary. It is therefore suggested that the process which was measured in curves *c* and *d* of Fig. 10 was the diffusion of calcium ions through the annular space. This interpretation is strengthened by the following facts reported by Allen. The probability of 'complete fertilization' decreases (i.e. the number of cases with a very low finally observed rate of transformation increases) as the 'degree of stretching' increases (i.e. as the length of the cylindrical portion increases). This is in conformity with expectation since an increase in the diffusion path decreases the rate in the well-known manner. In addition, the greater compression and distortion of the eggs in narrow tubes will cause these to have a tighter fit with consequent decrease in cross-sectional area of the annular space.

A decision as to the alternatives of calcium action, discussed before, may now be made. The calcium ions cannot be merely the initiators but are necessary for the *carrying out of the response*. Furthermore, they cannot be present to any appreciable extent in the cortex and are not liberated in the process of activation as suggested by Moser (1939*a*). They must be available at any portion of the surface where the reaction takes place. We can formally represent the situation by the following bimolecular reaction scheme, where M represents the component in the surface undergoing transformation.



or



In the presence of excess of calcium the reaction proceeds as a first-order autocatalytic process. When calcium is limited the rate will depend on its concentration which, in the capillary experiments, is limited by diffusion. This is then the rate-determining step. In the absence of calcium no reaction takes place.

Curve *b* in Fig. 10 represents an intermediate case. This was a moderately elongated egg (188μ) and consequently has only a portion ($<2/3$) of its surface obstructed. Although the vital information about the initial slope is missing, and it is impossible to say whether fertilization was equatorial or not, the change of slope is significant. While in the middle portion the rate is decreasing with diffusion as determinant, as the wave approaches the free end and reaches the region of unlimited calcium supply the autocatalytic mechanism takes over.

THE NATURE OF THE PRIMARY CHANGE

Some indications as to the nature of the change, as distinct from its propagation mechanism, emerge from this analysis. Since calcium enters the egg at all points along the response region and only when the response reaches any point, an increase in permeability of the surface must be involved. One might tentatively suggest that it is this change in permeability which constitutes the primary process of activation. Spermatozoa or any of the artificial agents can then be considered to effect an increase in permeability at the site of action which allows calcium to enter and convert the surrounding surface components into more permeable products. The initial increase in permeability is a sufficiently unspecific event to be caused by a large variety of agents which, with a surface of complex constitution, could effect the change by a variety of mechanisms. Whether the change is temporary or permanent is difficult to say since other processes such as membrane elevation and hardening supervene after a short time. The cortical granule transformation would then be considered synchronous with or immediately following the primary change. (See discussion by Sugiyama, 1953.) If the 'dying out' of the response in the region of low calcium concentration were a genuine 'death', i.e. that after an appropriate time interval the re-supply of calcium to the site would not result in a regeneration of the response, then the temporary nature of the response would be established. The published evidence by Allen points to this but does not permit a decision between it and the alternative of closure by a secondary process to be made. It is possible that this change plays a part in the block to polyspermy.

There is considerable information both on permeability and the role of calcium in activation and subsequent processes, but since most of it refers to experimental conditions very different from those considered, it would be inappropriate to discuss these here. However, it is worth pointing out that the eggs of some marine invertebrates have been shown to exhibit no measurable increase in permeability to water at fertilization. This is not incompatible with the mechanism suggested here. Firstly, there is abundant evidence from tracer work that transport across membranes, both active and passive, can be highly specific. It is clear that the conditions for permeability to positively charged calcium ions would be very different from those for neutral water molecules. Secondly, the change may be of short duration, sufficient for the passage of catalytic quantities of calcium, but not long enough for significant amounts of water to be detected by the methods employed.

Some of the results of Sugiyama (1953) possibly raise another aspect. Eggs of *Strongylocentrotus pulcherrimus*, pressed between cover-slip and slide into the shape of disks, were exposed to solutions of various activating agents for a short time. Sugiyama divides these agents into two classes, those having a 'propagating nature' and those having a 'non-propagating nature'. With the former the cortical granule breakdown, initiated in the exposed equatorial region, continues over the whole surface, with the latter it is confined to the exposed region. He suggests that the non-propagating agents affect the granules directly without initiating the (invisible) 'fertilization wave'. As no rate date are available, it is impossible to say whether there are real differences between agents or whether it is merely a time-scale difference. Allen (1954, p. 422) in similar partial exposure experiments on elongated *Psammechinus* eggs also describes a class of non-propagating agents but finds that others produce a wave-like breakdown which is slower than the normal fertilization response. Only one of the agents used (periodate) appears to initiate a response identical with that initiated by spermatozoa—if anything it is more effective. There is therefore a suggestion of a gradation of effects as an alternative to the idea of different mechanisms.

Differences in initial rate and total time between one experiment and another or between different activating agents could be caused by differences in the intensity and extent of the initial permeability change which is a function of the agent as well as the egg. In an autocatalytic equation the initial amount of catalyst as well as the specific rate constants determine the kinetics of the process. The state of maturity of the egg and its treatment before fertilization would influence mainly the internal specific parameters, while the nature and concentration of the activating agent or the age of the spermatozoon would influence mainly the initial parameters of the system. Species specific differences must be added to this. In view of this and the paucity of consistent experimental information on the nature of the substances involved it would be unfruitful to discuss these changes on the molecular level.

SUMMARY

The kinetics of the dark ground cortical change in fertilized sea urchins has been analysed. In normal eggs of *Psammechinus miliaris* the change appears to obey an autocatalytic mechanism.

The evidence from artificial activation suggests that the initiation of the response is caused by a relatively unspecific event.

The critical role of calcium is considered in relation to the evidence from eggs fertilized in capillary tubes. This suggests that calcium ions are not concerned with the initiation but with the propagation of the response.

The primary change in activation may consist of an increase in permeability at the site of initiation.

My thanks are due to Lord Rothschild, F.R.S., Prof. M. M. Swann and Prof. C. H. Waddington, F.R.S. for helpful discussion and criticism; and to the Nuffield Foundation for a grant.

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INHIBITORS OF TYROSINASE

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INTRODUCTION

The blood and tissues of most insects contain tyrosinase, together with a suitable substrate. When blood is shed or the tissues damaged, enzyme and substrate react and eventually produce melanin; why do they not react in the intact animal? The oldest theory was that they were held apart by some structural organization of the cytoplasm and that they only came together when this was destroyed. This explanation was first questioned by Graubard (1933) as a result of his investigations of the tyrosinase content of body-colour mutants in *Drosophila*. He found that he could obtain an extract containing an active tyrosinase by squashing larvae or pupae in water, but that if he ground them to a fine paste with sand most of the activity was lost. He concluded that there must be some inhibitor of tyrosinase inside the cells which was set free when they were broken open. By incubating the material for about 6 hr. in chloroform vapour the inhibitor seemed to be itself destroyed, so that grinding with sand yielded an active extract. Graubard's ideas were extended by Dennell, who suggested that the inhibitor might be a dehydrogenase system, because it was put out of action by chloroform; in later papers he confirmed this and identified the enzyme concerned more precisely. The final form of Dennell's views was summarized by Wigglesworth in the 1950 edition of his text-book as follows: 'Oxidation is apparently prevented by a dehydrogenase (acting upon glucose as a hydrogen donator) which reduces the oxidation-reduction potential of the blood. There is a rise in the oxidation-reduction potential just before pupation; tyrosinase can thus act upon tyrosine with the formation of DOPA.' Dennell's theory of inhibition has been extended to *Carcinus* by Krishnan (1950) and to Echinoderms by Jacobson & Millott (1953).

Another scheme, in which the inhibitor is supposed to be sulphydryl groups rather than glucose dehydrogenase, has been suggested for vertebrate melanophores by Figge (1940). Figge supposes that tyrosinase activity is maximal at certain oxidation-reduction potentials and that it can be inhibited by a shift from the optimal value in either direction, being almost completely inhibited by glutathione or cysteine. This theory was further elaborated by Figge & Allen (1941), who claimed that the inhibition could be removed by the action of various substances such as oestrone or methylene blue. Rothman, Krysa & Smiljanic (1946) claimed that macerated human skin contains an inhibitor of tyrosinase, which they supposed to be a sulphydryl compound. Hackman (1953) suggested that sulphur is absent from the cuticular proteins of insects because it would inhibit tyrosinase.

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THE MODE OF ACTION OF TYROSINASE

In spite of the general acceptance of these theories, reversible inhibition of tyrosinase has never been demonstrated *in vitro*, and it is not certain that the inhibitors suggested would have the effect required. This is clearly a critical point, and I shall therefore begin by examining some aspects of current knowledge about the action of tyrosinase to see how the proposed inhibitors might be expected to act. The subject has recently been reviewed by Dawson & Tarpley (1951) and by Lerner (1953).

Tyrosinase is a strictly aerobic enzyme, in that it can only catalyse the transfer of hydrogen to molecular oxygen. If amino-acids or ascorbic acid (Keilin & Mann, 1938) are present in excess they will reduce the *o*-quinones formed in the first stage of the reaction, becoming themselves oxidized in the process; the dihydroxyphenol formed by reduction of the *o*-quinone is then oxidized again by the enzyme, and the cycle is repeated. The final oxidation and polymerization of the aromatic substrate to form melanin cannot occur until all the amino-acid or ascorbic acid has been oxidized. Reaction mixtures containing ascorbic acid remain colourless, but with amino-acids a bright red colour develops, due to a stable condensation product between *o*-quinone and the amino-acid (Jackson & Kendal, 1949). Ascorbic acid or amino-acids cannot be said to 'inhibit the oxidation of tyrosine' (Dennell, 1947) since tyrosinase is in fact fully active in the presence of these reagents. Kendal (1949) has shown experimentally that tyrosine is oxidized in the presence of ascorbic acid to yield 3-4-dihydroxyphenylalanine (DOPA) which accumulates in the reaction mixture. The cycle involves continuous consumption of oxygen with the oxidation of ascorbic acid or amino-acid; there is no true reversal of the reaction in the sense of catechol donating hydrogen to the oxidized form of the substrate.

DESTRUCTION BY USE

Tyrosinase is unusual among enzymes in that it is destroyed by use; a given quantity of enzyme can only catalyse the uptake of a given quantity of oxygen (Ludwig & Nelson, 1939). The rate of destruction is higher for some substrates than others; phenol, catechol or methylcatechol, for example, having a strong effect, but tyrosine or tyramine hardly any. Initial concentration of substrate is not important; a small concentration which is continually reduced again by an excess of amino-acid or ascorbic acid has as much effect as an equivalent amount added all at once. The effect is not proportional to the concentration of poisonous end-products, since the rate of destruction is not decreased if quinones are removed as fast as they are formed by carrying out the reaction in the presence of benzene sulphinate (Bordner & Nelson, 1939).

INHIBITION IN INSECTS

We may now turn to a critical examination of the evidence in favour of the existence of inhibitors of tyrosinase in insects. The procedure used by Graubard to prepare his extracts was as follows. Larvae or pupae of *Drosophila* were ground with sand for about 15 min. in a small volume of chloroform water, and the mixture shaken in air for 1 hr. It was then diluted with phosphate buffer at pH 8·0 and centrifuged. This yielded an extract almost without activity. If the material was killed in chloroform and allowed to stand in chloroform vapour for an optimum period of 6 hr. an active extract was obtained. Crushing or light grinding without sand yielded an extract of intermediate activity.

Such a lengthy procedure would allow ample time for destruction by use, and the suspicion arises that the lack of activity was due to destruction of the enzyme rather than to the liberation of inhibitors. A simple hypothesis on these lines will in fact account for all Graubard's observations. Suppose that both enzyme and substrate are concentrated mainly in the cuticle, and that blood and fat-body contain abundant amino-acids or other reducing substances. When whole larvae are ground to a paste and shaken in air tyrosinase will act on its aromatic substrate many times over, thus suffering maximum destruction. If no sand is used the aromatic substrate is not set free from the cuticle and the small quantity of enzyme present in the blood and tissues is not much deactivated, aromatic substances in the blood being mainly in the form of tyrosine or DOPA, which are harmless. If larvae are allowed to blacken in chloroform vapour the aromatic substrate is oxidized *in situ* where it is not accessible to the reducing agents, so that it is only oxidized once, and the enzyme is not so completely destroyed. This amounts to the suggestion that the effect observed was not reversible inhibition but progressive destruction, and that the agent responsible was the natural aromatic substrate set free from the cuticle, and not a specific inhibitor set free from the cells. I will examine the evidence in favour of this hypothesis under several headings.

ACTIVITY OF FRESH EXTRACTS

Freshly prepared extracts are active whether they are ground with sand or not. If *Calliphora* larvae are crushed in a mortar into phosphate buffer and a sample is decanted, it is not appreciably active in oxidizing tyramine when tested in a Barcroft manometer. If the crushed remains are then ground with sand to a fine paste and another sample decanted, the activity is very much increased (see Fig. 1). In another experiment larvae of wild-type *Drosophila melanogaster* and mature (crop-empty) larvae of *Calliphora erythrocephala* were ground with sand to a fine paste, diluted with 0·1 M phosphate buffer at pH 8·0, and spun in a hand centrifuge, the whole operation lasting about 15 min. Samples were then tested in a Barcroft manometer, using excess tyramine hydrochloride as substrate, as in Graubard's experiments. It is convenient to quote the results in terms of q_{O_2}, meaning the number of cubic millimetres (μl.) of oxygen consumed per milligram live weight per hour. The *Drosophila* extract had a q_{O_2} of 0·8 and the *Calliphora* of 10·6, both at 25° C. Graubard's mean values for wild-type *Drosophila*, measured at the same pH and a temperature of 20·5° C., were 2 for larvae treated for 6 hr. with chloroform vapour, and 0·07 without chloroform.

SUBSTRATE AND ENZYME ARE CONCENTRATED IN THE CUTICLE

This was verified only on *Calliphora* larvae. Larvae were squashed one at a time on a glass plate by running over them with a rubber roller. This gave two fractions, 'skins', consisting of cuticle with some epidermis and muscle shreds attached, and 'blood and guts', consisting of viscera, fat-body and blood. The activity of each was tested separately. The material was ground with sand in phosphate buffer at pH 7·0, spun in a hand centrifuge to remove sand and shreds of tissue, and a sample taken into the Barcroft manometer. After equilibrating for temperature, oxygen uptake was recorded until it nearly ceased; excess tyramine hydrochloride was then added from a hanging tube and oxygen consumption measured again. The two fractions could not be weighed separately, so that only measures of total activity can be obtained, but these do in fact give interesting information. Before addition of tyramine the 'blood and guts' usually, but not always, take up more oxygen than the 'skins'. After addition of tyramine, the 'skins' always take up oxygen more rapidly; 'blood and guts' are often inactive. The preliminary oxygen consumption of the 'blood and guts' is always slow, although it may be large; that of the 'skins'

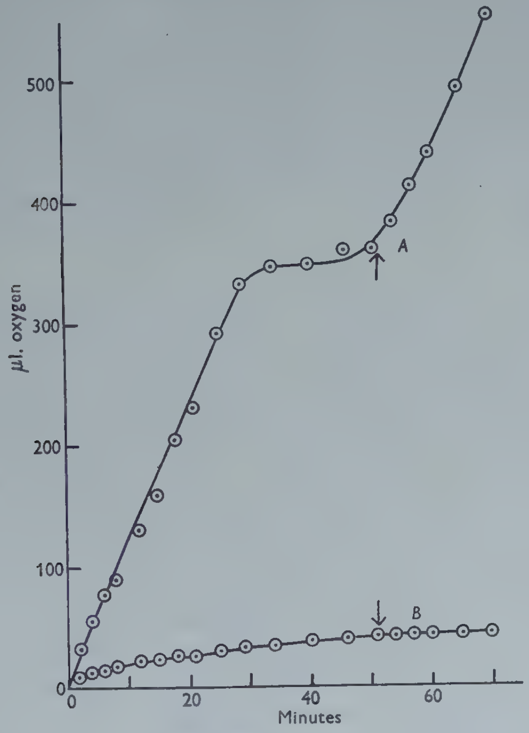

Fig. 1. Effect of crushing or grinding *Calliphora* larvae on tyrosinase activity. 1·4 g. mature *Calliphora* larvae crushed into 20 ml. phosphate buffer at pH 8·0. 5 ml. sample decanted (curve *B*). Remainder ground with sand, and another 5 ml. sample decanted (curve *A*). In both curves tyramine hydrochloride added at arrow.

is more rapid and ends more suddenly. This suggests that the 'skins' are richer in both enzyme and aromatic substrate, and that the extended oxygen consumption sometimes shown by the 'blood and guts' is due to the presence of amino-acids and other reducing substances. Typical curves are shown in Fig. 2.

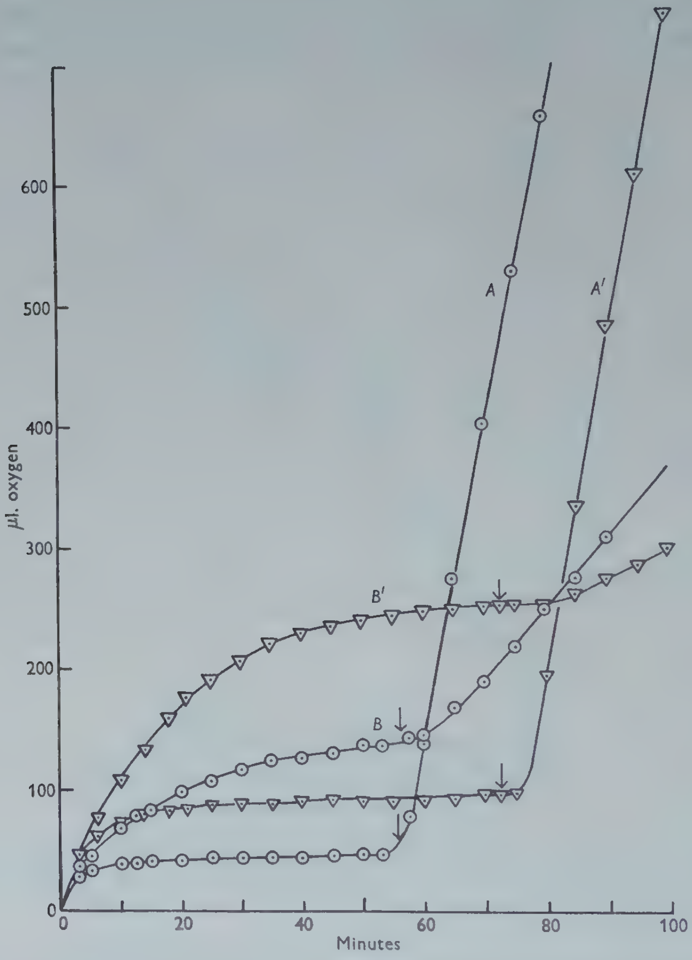

Fig. 2. Relative tyrosinase activity of 'skins' and 'blood and guts'. *A*, oxygen uptake of 'skins' of *Calliphora* larvae ground with sand; *A′*, the same for white pupae; *B*, oxygen uptake of 'blood and guts' of *Calliphora* larvae; *B′*, the same for white pupae. In both pairs of curves, excess tyramine hydrochloride added at arrows.

The activity of blood alone can be measured rather more exactly. By pricking the nose of a larva a drop of clear blood can be obtained; 1 g. of clear blood can be obtained from about a hundred larvae. This was diluted to 5 ml. with phosphate buffer at pH 7·0 and its activity measured, using tyramine. Taking samples on successive days from a large population of larvae all of about the same age, it was found that the activity of the blood was very small in mature (crop-empty) larvae;

on 2 successive days the q_{O_2} was 0·015, 0·02. On the third day the larvae started to pupate; taking a mixed sample from white pupae and late larvae the q_{O_2} was found to have risen to 0·09, all at 25° C. The highest value is still some hundred times lower than that for a typical crop-empty larva ground with sand. No measurement was made of the concentration of amino-acids in the fat-body or blood, but free amino-acids are present in the blood of *Calliphora* larvae (Finlayson & Hamer, 1949) and to judge from Levenbook's (1950) results on *Gastrophilus* they are probably in fairly high concentrations. In *Drosophila* the effect of reducing substances in increasing oxygen consumption and so contributing to the deactivation of tyrosinase is likely to be aggravated by the presence of immature larvae in the sample. Ohnishi (1953) has shown on freshly prepared extracts that the tyrosinase content of immature *Drosophila* larvae is very low and that it increases sharply just before pupation. A population of mixed ages such as Graubard and I have used will therefore contain many immature larvae which contribute amino-acids but little enzyme.

EFFECT OF THE NATURAL SUBSTRATE ON TYROSINASE

This was investigated only on *Calliphora*. Extracts prepared from mature larvae ground with sand as before were made to consume a small excess of oxygen by adding glycine or ascorbic acid, and were then tested against controls, using tyramine. Consumption of even a small amount of excess oxygen, of the order of size observed for example in 'blood and guts' preparations before addition of substrate, has a marked effect on the activity of the enzyme. In one example a brei of crop-empty *Calliphora* larvae had a q_{O_2} of 13·3. A duplicate sample, made to consume 4·4 mm.^{3} of oxygen per mg. by means of ascorbic acid, had a q_{O_2} of 6·9, both at 25° C. and pH 8·0. In a similar experiment on white pupae the q_{O_2} of the fresh extract was 3·9, and fell to 1·2 after consuming 4·38 mm.^{3} oxygen per mg.

The conclusions from these experiments are supported by a re-examination of Graubard's results. He found that when extracts of unchloroformed larvae acted on tyramine the rate of oxygen consumption fell as the reaction proceeded. This suggests progressive destruction. In preparations which showed this effect it was often noticed that the extract turned blue; the blue pigment was reduced on standing. It was less conspicuous when no sand was used in the grinding, and was never found after prolonged (12 hr.) treatment with chloroform vapour. A similar blue or purple pigment always appears in extracts of fresh *Calliphora* larvae, and it seems probable that it is formed by reaction of *o*-quinones with proteins or amino-acids; it is not formed if the larvae are allowed to blacken before being ground, or if tyrosinase is first inhibited with NaCN. The absence of blue pigment after light grinding or prolonged chloroform treatment suggests that both these treatments prevent the coming together of aromatic substances and amino-acids.

Graubard observed that extracts of *Tenebrio* larvae did not show any progressive loss of activity when tested with tyramine. This I have confirmed. It is also remarkable that as a rule no blue colour develops on standing; in the few preparations in which a blue colour did appear it was paler than that of a *Calliphora* extract of comparable concentration. All this suggests that the concentration of aromatic substrates is low in *Tenebrio* cuticle, agreeing with the fact that the cuticle darkens very slowly after moulting.

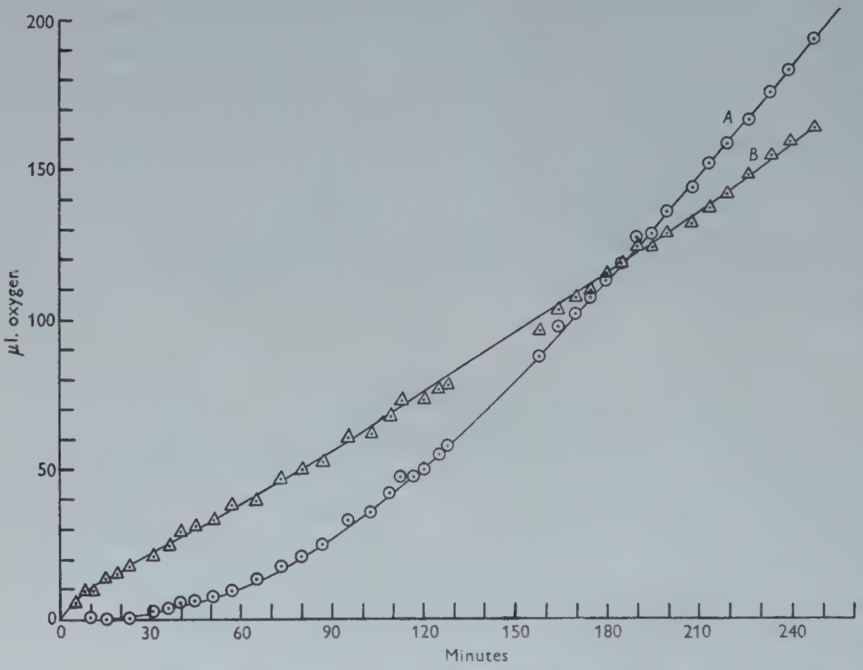

Fig. 3. Oxygen uptake of extract of *Tenebrio* larvae in presence of excess tyramine hydrochloride with (*B*), and without (*A*) methylcatechol. *A*, larval extract with 0·0042 g. tyramine hydrochloride (equivalent to 1120 μl. oxygen); *B*, the same, with addition of 0·0002 g. 4-methylcatechol (equivalent to 45 μl. oxygen).

The tyrosinase of *Tenebrio* extract is very sensitive to destruction by methylcatechol. In one experiment 4-methylcatechol equivalent to $3{\cdot}2 \times 10^{-3}$ time the weight of mealworms present in the extract was added to the extract and allowed to oxidize to completion before testing against tyramine. This treatment reduced the q_{O_2} from 1·9 to 0·7, both at 25° and pH 7·0. In another experiment (fig. 3), the addition, at the beginning of the run, of methylcatechol equivalent to $3{\cdot}5 \times 10^{-3}$ time the weight of larvae present in the extract reduced the final q_{O_2} from 1·0 to 0·57, although the sample with the methylcatechol started at a higher rate of oxygen consumption.

EFFECTS OF CHLOROFORM AND METHANOL

If freshly ground preparations of *Calliphora* larvae contained an inhibitor of tyrosinase which was destroyed by chloroform or methanol, it should be possible to increase the activity by adding these substances. I found, however, that the activity of a fresh extract of crop-empty *Calliphora* larvae was practically unaffected by the addition of methanol to give a final concentration of 0·1 M. A concentration of 0·2 M had a considerable effect in depressing the activity, but the readings were probably affected by the vapour pressure of the methanol. The effect of chloroform was tested by shaking the extract with excess chloroform, which was removed by evacuation before testing. This treatment considerably decreased the activity.

I was never able to repeat on *Calliphora* Graubard's experiments on *Drosophila*; exposure to chloroform vapour before grinding always reduced the activity. In one experiment two similar batches of crop-empty larvae were taken; one was exposed to chloroform vapour for 6 hr. and the other was kept during that time in a refrigerator. At the end of the 6 hr. both were ground with sand in buffer at pH 7·0, centrifuged, and tested with excess tyramine hydrochloride. The q_{O_2} of the control was 2·0; that of the chloroformed batch 1·06. In a similar experiment extending over 12 hr. the q_{O_2} of the control was 1·4, and that of the chloroformed sample 0·3. Blackening, or rather browning, of the larvae in chloroform vapour is complete within about 3 hr., but it usually remains patchy, affecting only the front end and parts of the rear end.

DEHYDROGENASES

If the effect of chloroform vapour is not specific, but is due to the fact that it kills the larvae without gross damage to their structure, then there is no reason, *prima facie*, to suppose that dehydrogenases are concerned in inhibiting tyrosinase in the intact insect, and it is worth examining the evidence again.

Agrell (1949) has shown that crushed pupae of *Calliphora erythrocephala* yield an extract capable of reducing methylene blue in the absence of oxygen. The rate at which the dye is reduced increases to a peak just after the time of darkening of the puparium, falls later in pupal life, and finally rises to another peak of about double its previous maximum just before the imago emerges. Agrell investigated the specificity of the enzymes concerned, using minced tissue washed free of substrate, and found that malic, succinic, citric, lactic and glutamic acids were all oxidized fairly rapidly, and glucose more slowly. The failure to obtain reduction of methylene blue by the blood of *Sarcophaga* larvae reported by Dennell (1947) is probably due to the fact that the blood was freely exposed to the air; for the same reason his estimates of the oxidation-reduction potential of the blood in the same paper are open to doubt.

So far the evidence seems clearly to indicate the presence of dehydrogenases, but interpretation of the classical tests for the presence of dehydrogenases is complicated by the presence of the tyrosinase system. The tyrosinase system will continue to consume oxygen as long as the supply of amino-acids or other suitable reducing substances lasts; where a local deficiency occurs the aromatic substrate will remain in the oxidized state, and will eventually blacken. In a test-tube full of brei left to stand in air a pink layer develops at the surface, which later turns purple and finally black, the rest of the tube remaining white. If the tube is shaken, the contents may become pink all through, but on standing the colour disappears again except at the surface. An artificial system containing catechol, glycine and a crude preparation of tyrosinase from mushrooms behaves in the same way. The behaviour of artificial systems with redox dyes is interesting. A mixture of catechol and tyrosinase will not bleach tolylene or methylene blue even if evacuated, but if excess glycine is added, the mixture, on standing, will reduce tolylene blue at the bottom of the tube. If the tube is evacuated the blue colour is discharged completely; this can be repeated many times. If a solution of tolylene blue in nearly oxygen-free water is injected by pipette into the lower part of the tube where the mixture has become colourless, the blue disappears and at the same time the cherry red colour of the glycine-quinone condensation product reappears. The reducing agent thus appears to be the glycine-quinone compound; this can be confirmed in another way. If the tube is evacuated immediately after mixing the ingredients no reduction of the tolylene blue takes place, but on admitting air there is a rapid fading of the blue as the pink colour develops. Thus not only is the reaction product of glycine and quinone a stronger reducing agent than either component separately, but it also differs from the simple catechol-tyrosinase system in that it will donate hydrogen to acceptors such as tolylene blue and not to molecular oxygen only. In more exact experiments, using a comparator, it was found that the artificial glycine-quinone-tyrosinase system cannot reduce methylene blue even *in vacuo*. The natural system present in *Calliphora* brei is more powerful, and will rapidly reduce methylene blue *in vacuo*; when air is admitted both blue and pink coloration start to develop at once.

The reduction of tolylene blue by the artificial glycine-quinone system is not affected by 2 M methanol, 0·1 M sodium malonate, or by saturation with chloroform or phenyl urethane. If sodium cyanide to give a final concentration of 0·01 M is added after the pink colour has developed it has no effect, although if it is added at the same time as the tyrosinase, reduction of the dye is prevented. From this it appears that once the red glycine-quinone compound has been formed, reduction of the dye does not require the presence of any enzyme. The behaviour of a brei of mature *Calliphora* larvae is similar. To investigate it a series of Thunberg tubes was set up containing a brei made from crop-empty *Calliphora* larvae in 0·2 M phosphate buffer at pH 7·0, coloured with methylene blue. Various reagents were added, and the tubes were then evacuated. It was found that excess chloroform or phenyl urethane or 0·1 M sodium malonate had no effect on the rate of bleaching. Tubes containing 0·01 M sodium cyanide bleached more slowly than the controls, although the difference was slight. After admission of air, tubes containing cyanide did not blacken; the rest formed a black layer at the surface. The brei used in these experiments was prepared by grinding maggots with sand in a mortar, a process which lasted about 10 min. During this time the brei turns bright pink, and it is probably this pink compound which is responsible for the reduction of the dye. If development of the pink compound is prevented by grinding the maggots in buffer containing 0·01 M sodium cyanide, the power to bleach methylene blue is almost lost, the time required being increased by some 10 times over the unpoisoned control. These experiments suggest that, as in the artificial system, reduction of the dye is brought about by an oxidation product of the aromatic substrate, and that it is not enzymic.

The effect of heat does not confirm this however. If the brei is heated on a water-bath at 90° C., and kept at that temperature for 3 min. the ability to bleach methylene blue is entirely lost; this is true even if the heating is carried out in an atmosphere of nitrogen. The artificial glycine-quinone system, on the other hand, may be boiled without affecting its power to reduce tolylene blue, although it changes colour from pink to purple. In view of the effects of enzyme poisons described above, or rather of their lack of effect, the result of this experiment is perhaps best interpreted as being due to destruction of the active compound. This interpretation is confirmed by the disappearance of the pink or purple colour; as the temperature of the brei rises above about 70° C. the pink colour fades and the proteins flocculate, leaving a creamy white curd. This is particularly conspicuous in a brei that has previously been aerated until it is a deep purplish pink colour.

The catalytic properties of oxidation products of the tyrosinase system were investigated further by the use of the Barcroft manometer. A mixture of one equivalent of catechol to two of glycine in the presence of a crude mushroom extract absorbed two atoms of oxygen per mol. catechol, as reported by Jackson & Kendal (1949). If excess glycine was added it was oxidized at a steady rate. Sodium cyanide up to a final concentration of 0·01 M had no effect on the oxidation of excess glycine if it was added after the first reaction was complete; if added at the beginning of the reaction it prevented oxygen uptake altogether. If more catechol was added to the system after the addition of 0·01 M sodium cyanide it was only slowly oxidized at pH 7·0. Tyramine was oxidized before the addition of cyanide but not after; glucose or succinate were not oxidized at all. An extract obtained by centrifuging maggot brei, if allowed to oxidize to completion and then poisoned with sodium cyanide, did oxidize tyramine, provided that the concentration of cyanide was not greater than 0·001 M. At a concentration of 0·01 M cyanide, oxidation of tyramine was very slight; about the same as in controls to which cyanide had been added before grinding the maggots.

The experiments described above show that in the presence of excess amino-acids or other suitable substrates, the tyrosinase system is capable of bleaching dyes, and that it is not necessary to invoke dehydrogenases to account for the bleaching of methylene blue by blood or by the endocuticle. Fluctuations in the rate of bleaching methylene blue with the age of the larvae, as reported by Agrell (1949), or fluctuations in the 'cyanide-insensitive respiration' as reported by Dennell (1949) are probably due, in part at least, to fluctuations in the concentration of aromatic compounds in the blood and tissues. On the other hand, the tyrosinase system is probably not responsible for the increase in oxygen consumption observed by Dennell after adding glucose to minced tissue, nor for the effect of succinic acid and other such substrates in accelerating the bleaching of methylene blue as observed by Agrell, since no action of this kind is shown by artificial preparations of known composition. It is indeed very probable that the tissues of insects should contain enzymes capable of oxidizing succinic acid or glucose, and if present they would probably contribute to the reduction of the tyrosinase system, but it does not seem that they have any important effect on the darkening of blood or tissues, either during life or after death.

CONTINUAL REDUCTION OF QUINONES *IN VIVO*

Even if there is no true inhibition, the possibility remains that tyrosinase activity *in vivo* is 'held in check by the reducing power of a dehydrogenase, tyrosine being reduced as rapidly as it is oxidized' (Dennell, 1944). Such a system, whether depending on a dehydrogenase or simply on amino-acids, would entail a high consumption of oxygen and the continual oxidation of some substrate. It can be shown that the oxygen consumption of a maggot is about doubled when it is squashed and starts to blacken, and this suggests that the tyrosinase system is not active in the intact animals, but only comes into action after injury. Sussman (1952) has found that the injection of small quantities of aromatic substances which would be oxidized by tyrosinase, or of known poisons of tyrosinase, has very little effect on the oxygen consumption of intact pupae of *Platysamia cecropia*, although both kinds of substance have their expected effect when added to a brei.

OXYGEN TENSION IN INSECT BLOOD

Barratt & Arnold (1911) found that no oxygen could be extracted from the blood of *Dytiscus marginalis* or *Hydrous piceus* with a vacuum pump, although the dissolved nitrogen was in equilibrium with air. If conditions are the same in the living insect, it seems possible that the action of tyrosinase is prevented by lack of oxygen. To test this idea I measured the oxygen tension in the blood of living insects by the bubble method of gas analysis described by Krogh (1911). Small bubbles were introduced into the body cavity, allowed to stay there for some hours, and then removed and collected by cutting open the insect under glycerine. The diameter of the bubble is measured under the microscope, the oxygen removed with alkaline sodium hydrosulphite, and the diameter measured again. After some practice, this method gave results repeatable to within about 2%. Controls were run on each batch, using either air or mixtures of air and nitrogen. The bubbles did not seem to harm the insects; some maggots pupated normally after having bubbles injected during their last instar. Twelve determinations on crop empty *Calliphora* larvae gave results between 15 and 22%, with a mean of 17·5%. Observations on other species gave similar results; larvae of *Pieris brassicae* in their final instar between 14 and 18%; *Tenebrio* adult (with the elytra removed) 17·3%. The only low values obtained were from some larvae of *Nymphula stratiotata* (Lepidoptera, Pyralidae) in which three individuals gave values of 1, 5, and 12%. This larva is aquatic, with a closed tracheal system, and the specimens used had been kept in a small tube of water on a warm day. The figures as a whole demonstrate that the oxygen tension inside an insect is not far removed from equilibrium with air; to prevent the action of tyrosinase a very much lower tension would be necessary.

If insect blood is drawn into a capillary tube it will blacken at the ends but not in the middle. If a tube is set up with a small bubble in the middle, it is possible by analysing the bubble after some hours to find out how far the tyrosinase system can reduce the oxygen tension before it ceases to function. This was tried with blood from adult *Periplaneta*, adult and nymphal *Locusta* and larvae of *Pieris brassicae*; in all, if the blood blackened it reduced the oxygen tension nearly to zero. Sometimes the blood did not blacken, and then there was no reduction in oxygen concentration.

These experiments show that it is not lack of oxygen that prevents blackening *in vivo*. They also show how completely the tyrosinase system can remove dissolved oxygen from the blood after death. It is undoubtedly this latter effect that is responsible for the absence of oxygen in blood recorded by Barratt & Arnold, who used samples of blood that had been standing for some time. The same effect will explain the observations of Dennell (1947) on the electrode potential of the blood of *Sarcophaga* larvae. In these experiments blood was collected into a small glass cell containing a platinum electrode, which was then connected to a standard half cell, and the e.m.f. of the whole measured. The blood showed a steady drift of potential on standing, reaching a level as low as +0·05 V. after some hours; this was probably due to the progressive disappearance of dissolved oxygen. Comparative measurements, all made 'as quickly as possible after withdrawal of the blood' showed a progressive lowering of the potential from the time that the larva left the food until the formation of the 'white pupa', when the potential rose suddenly. These fluctuations in potential correspond to fluctuations in the concentration of aromatic substrate in the blood. Kuwana (1937) described a similar fluctuation in the reducing power of the blood of silkworms, as assessed by the method of Hagedorn-Jensen for reducing sugars. The reagent is reduced by dihydroxyphenylalanine (Heller & Swiechowska, 1939); reducing power due to fermentable sugars was assessed separately. It was shown that a large part of the non-fermentable reducing substances disappeared on standing, their disappearance coinciding with blackening of the blood. Kuwana's own interpretation was that the non-fermentable fraction consisted of dihydroxyphenols, and that their disappearance was caused by oxidation; there seems to be no reason to doubt the correctness of this, although Dennell (1947) suggested that the non-fermentable reducing power was due to the action of dehydrogenases.

STRUCTURAL FACTORS

If tyrosinase is present in the tissues, and there is an adequate supply of oxygen and no specific inhibitor present, it seems that we must fall back on the original explanation of why living tissue does not blacken; that enzyme and substrate are not in effective contact. In the tissues it is not difficult to imagine some organization of the cytoplasm that would prevent enzyme and substrate coming together. In the blood, Dennell (1947) has described how tyrosinase is associated with granules inside some of the blood cells; it is to be supposed that these cells become permeable and allow enzyme to escape when blood is shed. Dilution of the blood with water accelerates darkening (Dennell, 1947; Ohnishi, 1953), probably because it promotes cytolysis. Dennell (1947) described a remarkable effect of methyl alcohol (and to a lesser extent of other alcohols) in promoting blackening of the cuticle; this may perhaps be explained as being due to disruption of the organized structure by the alcohol as it penetrates, without any too rapid inactivation of the tyrosinase.

INHIBITION IN OTHER GROUPS

The system present in *Carcinus*, as briefly described by Krishnan (1950), seems to be like that of insects, with similar fluctuations in the cyanide-insensitive respiration, which can probably be accounted for in the same way. The coelomic fluid of the echinoid *Diadema antillarum*, described by Jacobson & Millott (1953), behaves like the blood of *Sarcophaga* larvae as described by Dennell (1947). It darkens when shed, the darkening starting from granules in some of the amoebocytes; it is shown that tyrosinase is associated with the granules, and that an aromatic substrate is present in the amoebocytes as well. Darkening is accelerated by dilution with distilled water, and is accompanied by cytolysis of the amoebocytes; it is delayed by the addition of filtered coelomic fluid. The only particular in which this system seems to differ from that of insect blood is that the 'inhibitory' effect of coelomic fluid is destroyed by boiling, but this may be due to the removal of proteins.

INHIBITION BY SULPHYDRYL COMPOUNDS

Inhibition by sulphydryl groups cannot be of much importance in insect cuticles, but it might occur in arachnids or in the chorion of some insect eggs. In vertebrates, far from there being any evidence of inhibition, there seems to be a positive correlation between the activity of tyrosinase and the oxidation of sulphydryl groups; sheep from copper-deficient pastures, which cannot form tyrosinase, show delayed oxidation of the sulphydryl groups in their wool (Marston, 1952). The experimental evidence of inhibition by sulphydryl groups is weak; progress of the reaction was only followed colorimetrically, apparently in unstirred vessels (Figge, 1940; Figge & Allen, 1941). In some experiments there is little evidence that the reaction mixture contained free sulphydryl groups, although it probably did contain proteins and amino-acids (Rothman, Krysa & Smiljanic, 1946). Failure to darken in these experiments does not necessarily imply the inhibition of tyrosinase, but might have been due to continual reduction of the products of the reaction. I therefore investigated the effects of sulphydryl groups by adding reduced glutathione to mixtures of catechol with a crude tyrosinase from mushrooms, and measuring the oxygen uptake in a Barcroft manometer. The results depended on the relative concentrations of catechol and glutathione. One mol. catechol to one of glutathione absorbs three atoms of oxygen, suggesting that the catechol is oxidized in the normal way, taking two atoms of oxygen and that the glutathione is also oxidized, taking one more. Two, three, or four mol. of glutathione to one of catechol all require only one atom of oxygen per mol. of catechol, suggesting that the *o*-quinone combines with the thiol groups to form a compound which does not react further (Fig. 4).

The reaction between *p*-quinone and cysteine has been investigated by Kuhn & Beinert (1944) and by Burton & David (1952), and they agreed that the initial reaction is a direct condensation between the SH group and the aromatic nucleus; with *o*-quinone, two SH groups might be expected to react with each nucleus. The compound formed is evidently colourless, as the solutions remain uncoloured, although solutions containing two mol. of catechol per mol. glutathione turn red. A similar result was obtained with tyramine, using a dilute extract of *Calliphora* larvae instead of mushroom powder. At pH 7·0, one mol. tyramine normally requires 4·1 atoms of oxygen for complete oxidation. Addition of glutathione in equimolecular proportion or less results in uptake of one extra atom of oxygen per mol. glutathione. Two or three mol. of glutathione per mol. tyramine reduce the oxygen uptake to two atoms per mol. tyramine. In this instance it is perhaps doubtful whether the thiol groups can react directly with the aromatic nucleus; the same result would be obtained if the excess glutathione prevented further oxidation and polymerization of the dihydroxyindole formed by the initial oxidation of tyramine.

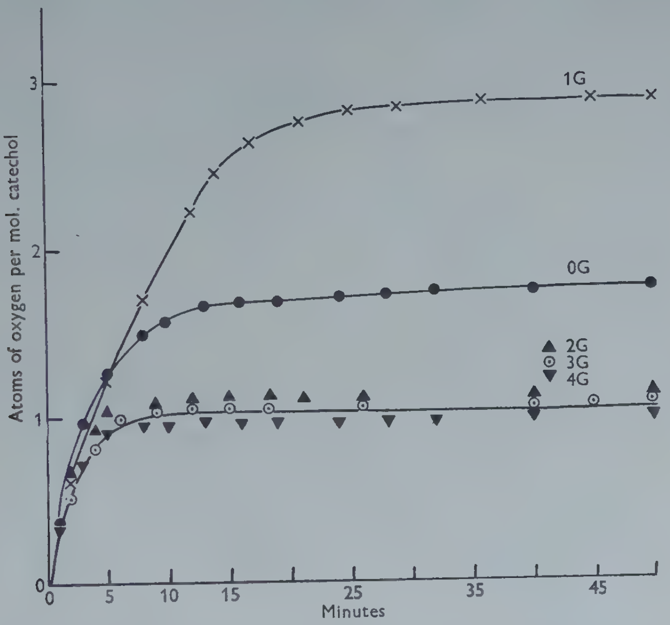

Fig. 4. Effect of reduced glutathione on the oxidation of catechol by mushroom powder. The letter against each curve indicates the relative molar concentration of reduced glutathione to catechol. Concentration of catechol $0{\cdot}2 \times 10^{-5}$ M in all.

In most of these experiments the absolute concentration of glutathione was $0{\cdot}2\,\mathrm{M} \times 10^{-2}$; in some it was as high as $1{\cdot}2\,\mathrm{M} \times 10^{-2}$. There was no evidence of any slowing of the reaction with catechol. With tyramine, the rate of oxygen uptake is reduced with increasing concentration of glutathione. Fig. 5 shows the rate of oxygen uptake of identical samples of *Calliphora* extract with various concentrations of tyramine hydrochloride and glutathione; the letters against the curves indicate the relative molar concentrations. The glutathione (G) appears to act by eliminating the later, more rapid, stages of the reaction; this is more likely to be caused by reaction with the substrate than by inhibition of the enzyme.

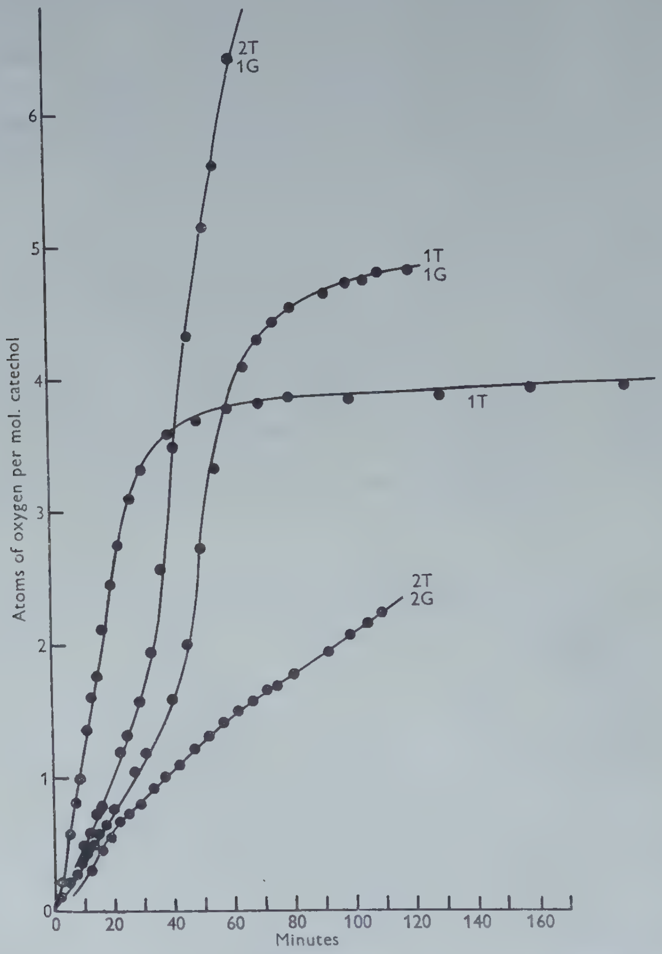

Fig. 5. Effect of reduced glutathione on the oxidation of tyramine hydrochloride by extract of *Calliphora* larvae. The letters against each curve give the relative concentration of tyramine hydrochloride (T) and glutathione (G), taking a unit (1 T, 1 G) of $0{\cdot}2 \times 10^{-5}$ M.

SUMMARY

1. It has been reported that if *Drosophila* larvae are ground to a fine paste with sand, the homogenate shows little tyrosinase activity, but that if the larvae are allowed to blacken in chloroform vapour before grinding, activity is increased.

2. This has been interpreted as showing the effect of an intracellular inhibitor, set free by rupturing the cells, but destroyed by chloroform. This inhibitor has been identified by previous authors as a dehydrogenase.

3. It is here suggested that the lack of activity of *Drosophila* extracts prepared with sand is due to destruction of tyrosinase as it oxidizes naturally occurring aromatic substrates.

4. It is shown that tyrosinase is destroyed by oxidizing the aromatic substrate present in the cuticle of *Calliphora* larvae, or by very low concentrations of homocatechol.

5. The aromatic substrate of *Calliphora* larvae is concentrated in the cuticle, and would be set free by fine grinding.

6. *Drosophila* or *Calliphora* larvae yield a more active extract when ground with sand than when simply crushed, provided that they are tested soon after grinding.

7. The tyrosinase activity of such extracts is not increased by chloroform or methanol.

8. The compound between *o*-quinones and amino-acids is capable of oxidizing ascorbic acid or excess amino-acid without the aid of an enzyme, and of simultaneously reducing methylene blue.

9. This reaction, rather than the activity of dehydrogenases, is probably responsible for most of the ability of damaged insect tissue to bleach methylene blue.

10. The blood of insects normally contains dissolved oxygen in equilibrium with the air.

11. The reaction involved in the blackening of insect blood may consume all the dissolved oxygen.

12. Previous observations on fluctuations in the oxidation-reduction potential of the blood of *Calliphora* larvae with age are probably due to changes in the rate at which oxygen is consumed by the blood after it is shed.

13. There does not therefore appear to be any valid evidence that tyrosinase is inhibited *in vivo* by the action of dehydrogenases. The absence of tyrosinase activity in undamaged tissue is probably due to the structure of the cytoplasm, which keeps enzyme and substrate apart.

14. Instances of the inhibition of tyrosinase reported in Crustacea and Echinodermata seem to be susceptible of the same explanation as in insects.

15. The supposed inhibitory effect of sulphydryl groups reported for vertebrate melanophores is shown to be due to the combination of sulphydryl groups with *o*-quinones, which prevents the formation of melanins.

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THE BREAKDOWN OF THE THORACIC GLAND IN THE ADULT INSECT, *RHODNIUS PROLIXUS*

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The thoracic or 'prothoracic' glands of insects, which are the source of the growth and moulting hormone of the larval and pupal stages, degenerate and disappear soon after the final moult. In the adult *Rhodnius* the large nuclei of these glands are at an advanced stage of disintegration within 24 hr. of moulting, and at 48 hr. they have completely disappeared (Wigglesworth, 1952). The object of the present work was to study the physiological control of this breakdown.*

THE JUVENILE HORMONE AND THE PERSISTENCE OF THE THORACIC GLANDS

If an extra larval stage (6th-stage larva) is produced in *Rhodnius* by the implantation of the corpus allatum from a 4th-stage larva into the 5th-stage larva, it will usually moult again after feeding (Wigglesworth, 1936). Presumably, therefore, these 6th-stage larvae retain their thoracic glands along with their other juvenile characters. This has been confirmed by dissection.

With the object of obtaining a series of intermediate stages, the corpus allatum from the 4th-stage larva was implanted into the abdomen of a number of 5th-stage larvae. The resulting forms ranged from normal adults with a small localized patch of larval cuticle over the site of the implant, through a series of more or less larval forms with enlarged and crumpled wings, to the usual 6th-stage type of larva (see Wigglesworth, 1936, pl. 5, figs. 12–16; and Wigglesworth, 1948, pl. 1, figs. 5 and 10–12). These intermediate forms were then fed. When it appeared (from the visible changes in the integument) that moulting had begun, they were dissected and the thoracic gland mounted. If they failed to moult they were fed again once or twice and finally dissected.

Those which approximate to the adult form (having only local patches of larval cuticle) show no sign of moulting and are found on dissection to have lost their thoracic glands.

Those approximating to the larval form (with varying degrees of enlargement of the wing lobes and of development of the genitalia) retain the thoracic gland cells, though in some the cells are reduced in number. After feeding, most of these insects moult. Moulting may proceed at the normal rate and in these the thoracic gland cells are numerous and active (Wigglesworth, 1952, fig. 6B); or moulting may be delayed and in some of these the thoracic gland cells appear to be reduced in number.

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* This work was begun jointly with Mr M. J. Wells, who carried out a few preliminary experiments on *Oncopeltus fasciatus*. The results are not reported here, but so far as they went they agreed with those obtained in *Rhodnius*.

But in some of the larval forms moulting fails to occur in spite of full and repeated feeds of blood. One 6th-stage larva, produced by the implantation of the corpus allatum from an adult *Periplaneta* in October 1950, was fed repeatedly until April 1954 when it was dissected. In those larval forms which fail to moult the thoracic gland cells are present but show no signs of secretory activity. The number of these cells is sometimes reduced, but often appears normal. The failure to moult presumably results from some other defect in the endocrine system: the thoracic glands are not activated.

These experiments confirm the assumption that the juvenile hormone ensures the persistence of the thoracic glands. This is clearly effected by humoral means. It remains to be proved that their breakdown in the adult is also brought about by humoral means.

THE HUMORAL CONTROL OF BREAKDOWN OF THE THORACIC GLANDS

By reason of their great size and lobulation it is quite easy to recognize the nuclei of the thoracic gland cells after transplantation to the abdomen. In many of the experiments to be reported the glands (together with the slender lobe of the fat body with which they are associated) have been implanted immediately below the integument of the abdomen in another insect. At a later stage this region of the abdomen has been examined in whole mounts or in section.

If the glands are removed from an unfed 5th-stage larva and implanted into the abdomen of another 5th-stage larva one day after feeding they are still intact when this insect moults to become adult, but they then break down, synchronously with the gland cells of the host, during the next 24 hr.

The same results are obtained if the thoracic glands from an unfed 4th-stage larva are implanted into another 4th-stage larva soon after feeding. They remain intact until the host has undergone two moults and become adult; they then break down during the following day.

If a 4th-stage larva receives a thoracic gland implant from an unfed 5th-stage larva, is allowed to moult twice to become adult and is then dissected at once, the transplanted thoracic gland is still present. The juvenile hormone of the 4th-stage larva has led to its preservation from an extra instar. The gland then breaks down synchronously with the glands of the host.

Conversely, if the thoracic gland removed from an unfed 4th-stage larva is implanted into a recently fed 5th-stage larva it breaks down as soon as the host becomes adult. In this case the implanted gland has disappeared after going through four moulting stages instead of the usual five.

Since the thoracic gland, both in its normal position and after transplantation to the abdomen degenerates within 24–48 hr. after the adult moult, it is clear that this breakdown must be humorally determined.

TIME OF ACTION OF THE JUVENILE HORMONE

We have seen that exposure to the juvenile hormone leads to the persistence of the thoracic glands. In the newly moulted adult the juvenile hormone is absent (Wigglesworth, 1948). It was therefore of interest to consider whether the glands can be preserved by exposure to the juvenile hormone at this stage.

The front half of the head was removed from 4th-stage larvae at 7 days after feeding, that is at a time when there is abundant juvenile hormone circulating in the blood, and they were then joined to newly moulted adults (through a small excision in the sternites) so that these received juvenile hormone in the circulating blood. The adults employed had moulted 2–6 hr. previously. They were dissected 5 days later and in all cases the thoracic glands had disappeared.

In other experiments the thoracic glands were dissected out from adults within less than 3 hr. after moulting and implanted into the abdomen of 4th-stage larvae at 7 days after feeding. The host was allowed to moult to the 5th stage and then dissected immediately. In none did the thoracic gland nuclei survive.

Clearly the thoracic gland of the newly moulted adult will not survive even in an environment containing abundant juvenile hormone. In order to ensure survival it must be exposed to the juvenile hormone during the preceding stage of growth leading up to moulting.

Will the gland survive if it is exposed to the juvenile hormone during the later stages of moulting? This was investigated by a modification of the technique previously employed (Wigglesworth, 1940) for switching over from adult development to larval development during the 5th stage. The 5th-stage larva was allowed to develop for 10 days or more at 25° C. The head was now left intact and a 4th-stage larva at 7 days after feeding joined to the abdomen.

The 5th-stage larvae were treated in this way at 10, 12, 14 and 17 days after feeding. The characters developed when they moulted were chiefly those of the adult (because development of most adult characters was already well advanced before the circulating juvenile hormone became effective), but the presence of juvenile hormone in these insects was proved by the development of partially larval characters in the abdominal cuticle in those operated upon at 10 and 12 days after feeding. In all of them the thoracic glands had completely disappeared by 3 days after moulting.

Clearly the thoracic gland cannot be protected from breakdown by exposure to juvenile hormone during the second half of the moulting period. It can be preserved only if 'metamorphosis' is prevented by the action of the juvenile hormone from an early stage.

THE FACTOR INDUCING BREAKDOWN OF THE THORACIC GLANDS

Unless the thoracic gland has been exposed to the juvenile hormone from an early stage in the moulting period it breaks down within 24–48 hr. after the insect becomes adult; and since this change occurs in transplanted glands it must be determined humorally. That is, there must be some humoral change in the newly moulted adult which induces breakdown. Will this humoral change in the adult at the time of moulting induce the breakdown of thoracic glands of other insects exposed to it?

5th-stage larvae at 1 day after feeding were transfused with circulating blood from 5th-stage larvae 22 days after feeding—and continuously throughout the period of the next 3 or 4 days while the latter insects underwent their moult to the adult stage. The experiment was performed in two different ways: (*a*) by joining the head of the 5th-stage larvae at 22 days after feeding to the abdomen of those at 1 day after feeding; (*b*) by joining the head of the insect at 1 day to the abdomen of the insects at 22 days. In both series the younger 5th-stage larvae were left for 3–10 days after their partners had moulted, and were then dissected. In none had the thoracic gland been induced to break down.

The same experiments were performed using 4th-stage larvae 1 day after feeding for joining to the 5th-stage larvae during the final days of moulting. The thoracic glands of the young adults disappeared as usual within 2 days after moulting; those of the 4th-stage larvae were not affected.

In another series of experiments the thoracic glands from unfed 5th-stage larvae were implanted into newly moulted adults within 3 hr. after moulting. These were dissected 7 days later but in none had the implanted glands been induced to break down.

We must therefore conclude that two factors are concerned in the breakdown of the thoracic glands.

(i) They must undergo some process of change (or 'metamorphosis') during the preceding moulting stage. Unless they have undergone this change they will not break down even when exposed to the environment in the newly moulted (or actually moulting) adult which induces the breakdown in its own thoracic glands.

(ii) They must then be exposed to some further humoral stimulus which occurs at the time of moulting to the adult stage. After experiencing this further stimulus the glands apparently break down in any environment.

At what stage in the moulting process does this second change come about? A long series of experiments was carried out in which the thoracic glands were removed from 5th-stage larvae at different stages in the moulting process and implanted into the abdomen of recently fed adults. The adults employed were usually about 10 days old. The results may be summarized as follows.

(i) Thoracic glands from 5th-stage larvae 7 days after feeding implanted; the adults dissected 13 days later. There were nine survivors. Thoracic gland cells had survived in all.

(ii) Glands from 5th-stage larvae 14 days after feeding implanted; the adults dissected 12 days later. There were six survivors. In all of these the thoracic gland cells were conspicuous and actively secreting (that is, with enormous nuclei and abundant cytoplasm). In most experiments there were mitoses and other signs of moulting in the adult cuticle overlying the implant. In at least one case these changes were general over the surface of the abdomen.

(iii) Glands from 5th-stage larvae 21 days after feeding implanted; the adults dissected 7 days later (the 5th-stage larvae of the same batch had all moulted by this time). Out of ten survivors, eight showed healthy nuclei surviving. This is well past the time when they would have broken down if they had been left in their normal position.

(iv) Glands from 5th-stage larvae at 24 days after feeding implanted. (Most of the other larvae in this batch had already moulted; these would probably have moulted within 24 hr.) Among twenty survivors dissected 5–9 days after the transplantation, the survival of the thoracic gland was established in seventeen.

(v) Glands were removed from adults at the time of moulting. In some the cuticle had just split and they were only part way out of the old skin, others had been out of the cuticle for a few minutes and others had moulted an hour or so previously. Among twenty surviving adults which received these implants three were dissected 4 days after implantation. In these some surviving nuclei of the thoracic gland could be seen, though many of them were in process of dissolution. Breakdown evidently proceeds more slowly in the newly transplanted gland. But in all the remainder (dissected 8–11 days after transplantation) the gland cells had degenerated—with the exception of one specimen (where the glands had been transplanted from an insect in the act of moulting) in which a number of apparently healthy thoracic gland nuclei remained intact.

Conclusions. Although it has not been possible by means of these experiments to fix precisely the moment at which the stimulus to breakdown is imparted to them, the results support the view that just around the time of moulting to the adult something happens to the thoracic glands which determines the subsequent breakdown of cells.

THE SOURCE OF THE STIMULUS INDUCING BREAKDOWN OF THE THORACIC GLANDS

If the adult *Rhodnius* is decapitated immediately after moulting the dissolution of the thoracic gland is not affected: breakdown is complete in 48 hr. But we have seen that the stimulus which determines this breakdown is effective probably during the act of moulting or very shortly before. A series of 5th-stage larvae were therefore decapitated at 23 days after feeding. The time of moulting can be recognized by the appearance of air beneath the old cuticle. There were seven survivors: one was dissected at 1 day after moulting and showed the thoracic glands at an advanced stage of breakdown; six were dissected at 2 or 3 days after moulting and in these the thoracic glands had disappeared.

One must conclude from these experiments that the stimulus to breakdown does not come from the brain, suboesophageal ganglion, corpus allatum or corpus cardiacum. In *Periplaneta* the prothoracic gland requires some 12–14 days for its complete degeneration. But if the corpus allatum is removed, the brain and corpus cardiacum being left intact, the breakdown of the prothoracic gland can be prevented (Bodenstein, 1953). Bodenstein has therefore suggested that the presence of the corpora cardiaca in the absence of the corpora allata is responsible for the maintenance of the prothoracic gland of the adult.

Degeneration of the thoracic gland in the adult *Rhodnius* is determined so rapidly, and the anatomical relations are such that it has not been feasible to repeat Bodenstein's experiment. As an alternative, the corpus cardiacum from the unfed 5th-stage larva, separated from the brain and the corpus allatum, was implanted into the abdomen of the 5th-stage larva 4–5 days after feeding. The resulting adult was then decapitated within a few minutes after moulting or, at latest, within 4–5 hr. In seven surviving adults the course of breakdown in the thoracic gland was not affected.

DISCUSSION

It is clear from these results that two factors are involved in the breakdown of the thoracic gland in the adult *Rhodnius*.

(i) It must undergo some invisible change or 'metamorphosis' resulting from a moulting stage passed in the absence of the juvenile hormone.

(ii) It must then experience some humoral stimulus at the time of moulting to the adult which induces in it an irreversible tendency to degenerate.

The nature of this second stimulus is unknown. But the phenomenon as a whole is exactly comparable with what happens in some other tissues of the insect at metamorphosis. For example, the larval fat body in *Drosophila* is conspicuous in the newly moulted adult but breaks down and disappears during the next 4 days (Wigglesworth, 1949). Indeed, at the moment of moulting, with or without metamorphosis, processes of many diverse kinds are set in motion, such as the darkening and hardening of the cuticle, the dissolution of unwanted muscles, and the discharge of the 'cement' from the dermal glands. These processes are presumably initiated by humoral stimuli but the source of the secretions concerned is unknown. Perhaps the neurosecretory cells which are so conspicuous in the thoracic and abdominal ganglia of *Rhodnius* and other insects may be concerned.

In general the results described agree with those reported by Bodenstein (1953) in *Periplaneta*. For Bodenstein also found that transplantation of larval corpora allata into young adult hosts does not prevent the breakdown of the prothoracic gland, and that larval prothoracic glands transplanted into old adult hosts fail to degenerate.

The most striking observation reported by Bodenstein was that removal of the corpus allatum from a newly moulted adult *Periplaneta* led to the persistence of the prothoracic gland and the moulting of the adult. No satisfactory explanation of this result has been found. But there is an observation on *Rhodnius* which may possibly have a bearing upon the problem.

If the thoracic glands removed from a 5th-stage larva 10 days after feeding (that is, at the height of their activity) are implanted into the abdomen of an adult female *Rhodnius*, this is induced to lay down a new cuticle—that is, to 'moult'. But the process of egg development is not inhibited; most of the available food is diverted to the ovaries, and the new cuticle is exceedingly thin and delicate. On the other hand, if the corpus allatum is removed by decapitation at the same time as the thoracic glands are implanted, the deposition of yolk is inhibited and the moulting adult now lays down a new cuticle of normal thickness (Wigglesworth, 1952). It may be that in Bodenstein's experiments, after the corpus allatum has been removed from a newly moulted adult the food materials are no longer diverted to the reproductive organs, the prothoracic gland resumes its activity and moulting occurs.

SUMMARY

The thoracic gland in *Rhodnius* breaks down and disappears within 48 hr. after the moult to the adult stage.

Two factors are involved: (i) The gland suffers some change as the result of going through a moulting stage in the absence of the juvenile hormone. (ii) It must then be exposed to some humoral action at the time of the adult moult.

The source and nature of the stimulus which operates at the time of the final ecdysis are not known. But when the gland has been exposed to these two changes it will rapidly break down even in an environment containing the juvenile hormone.

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PHOTO-KINESIS IN THE AMMOCOETE LARVA OF THE BROOK LAMPREY

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INTRODUCTION

Young (1935*a*) found that the ammocoete larva of the lamprey collect in the shaded half of a tank if the other half is brightly lit. He showed that the effect of bright light is to initiate swimming movements which are apparently at random, the ammocoetes entering the shaded part of the tank by chance and coming to rest there in the absence of further stimulation. This reaction, in which the response has no directional relation to the stimulus, was described as a photo-kinesis.

Hawes (1945) has shown that *Proteus anguinus*, a blind, neotenous, cave-dwelling urodele, reacts to light in a manner similar to that of the ammocoete larva. Hawes does not believe that the swimming movements of *Proteus* are truly at random, and has suggested that this might also be so in the case of the ammocoete. The meaning of the term kinesis is twofold (Gunn, Kennedy & Pielou, 1937). First, the direction in which the animal moves is at random. Secondly, in an ortho-kinesis the speed or frequency of movement is dependent on the intensity of stimulation, and in a klino-kinesis the frequency or amount of turning is similarly dependent. Young's results were published before the terminology was introduced, and the ammocoetes' response to light was subsequently classified as an ortho-kinesis by Fraenkel & Gunn (1940). But there has been no quantitative demonstration of an ortho-kinesis, no suggestion of a klino-kinesis, and now doubts have been raised by Hawes (1945) as to whether the swimming movements are really at random. An attempt was therefore made to give a quantitative demonstration of ortho-kinesis. Observations were also made on the behaviour of ammocoetes in a light gradient. These experiments could give misleading results if the ammocoetes had an inherent rhythm of activity. Young (1935*b*) has shown that their melanophores expand during the day and contract at night, the rhythm persisting in constant darkness and at a constant temperature. The possibility of a similar rhythm in locomotory activity was therefore examined before passing on to the other experiments.

MATERIAL

Ammocoete larvae of *Lampetra planeri* (Bloch) were collected from a stream at Snailwell, near Newmarket. Their sizes ranged from 80 to 150 mm. In the laboratory they were kept in running water, rotting leaves, and mud. The stock was replenished from time to time to make good losses by escape and accident.

LIGHT MEASUREMENTS

Light intensities were measured by an R.C.A. 931 A photomultiplier tube using a circuit designed by Dr R. H. J. Brown and Mr J. A. Popple in the Zoological Laboratory, Cambridge. The essential features of the circuit are given in Fig. 1. The 1300 V. d.c. supply is transformed from 200 to 250 a.c. mains, rectified and stabilized. The resistance of the photomultiplier decreases with increasing light intensity and automatically turns down the voltage supplied to it by altering the grid bias on the series valve. The voltage change is proportional to light intensity and is measured in the following way. The potential difference between the voltage applied to the photomultiplier and that of a selected point across the 1300 V. supply is passed, with suitable series resistors, across a 100 μA. meter, to give a full-scale deflexion in the dark. Two tappings were made from the high voltage supply, one covering the full range from bright light to darkness, the other giving a full-scale deflexion at low intensities. The photomultiplier was housed in a waterproof bottle enclosed in a metal case with an aperture over the cathode. The aperture was surrounded by a shield to give the cell directional properties. The photocell was calibrated, using Ilford neutral density filters, against a uniplanar tungsten filament substandard lamp supplied and rated by the British Thompson-Houston Co. Ltd. The intensities covered ranged from 10^3 to 10^{-3} metre candles (m.c.). No attempt was made to work at lower intensities, although this can be done by selecting suitable tapping points from the high voltage supply.

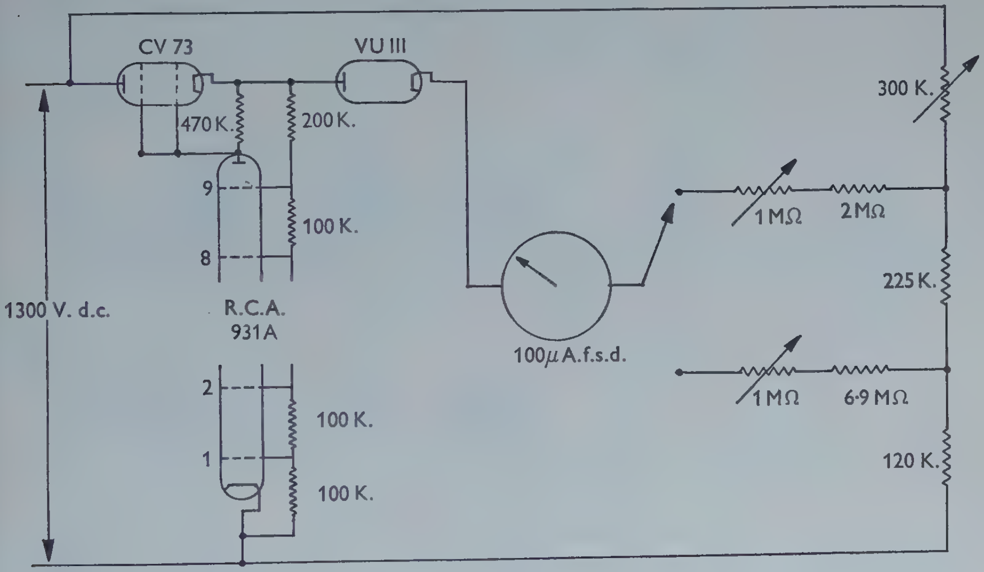

Fig. 1. Diagram of circuit used with photomultiplier.

RHYTHMICAL ACTIVITY

Methods

The activity of the ammocoetes was measured by an automatic recorder. Activity recorders for aquatic animals have been described by Szymanski (1914), Spencer (1929), Kalmus (1939), Roberts (1940) and Spoor (1941). The animal is either attached by thread to a lever which writes on a kymograph drum, or it displaces a vane, whose movement is indirectly recorded. The device used in these experiments was of the latter type. A celluloid vane was suspended in the experimental tank by work-hardened resistance wire placed so that a slight displacement of the vane would allow it to complete a circuit by coming into contact with a metal bar. To avoid the adhesion which occurs if appreciable current is passed through the contacts, the recorder was used, at the suggestion of Dr R. H. J. Brown, in conjunction with the circuit shown in Fig. 2. When the contacts meet they short out the grid bias on the valve, allowing it to conduct current to operate a relay. The relay operates a time marker which records single contacts on a 12 in. diameter smoked kymograph drum. Further relays and markers allowed every tenth and hundredth contact to be recorded. The drum revolved once every 24 hr. and a fourth marker gave half-hourly marks.

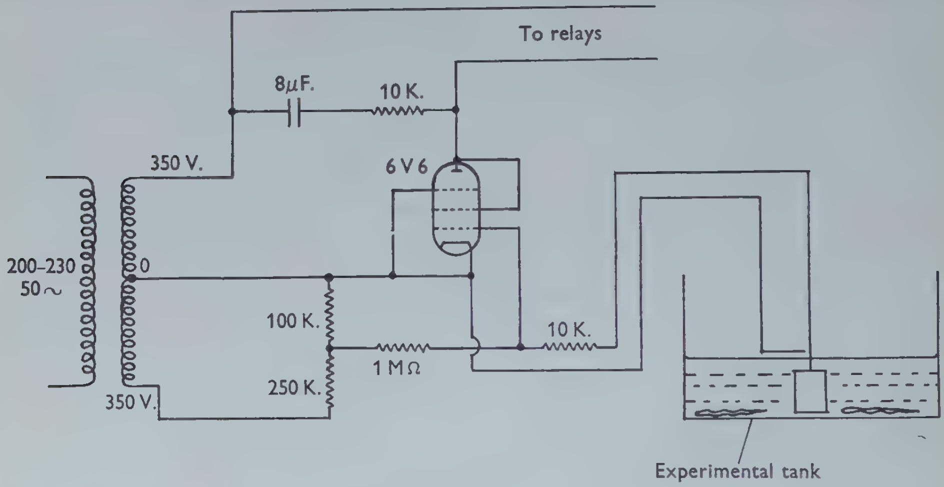

Fig. 2. Diagram of activity recorder and circuit.

Not every swimming movement is recorded. Although the chance of a movement being recorded depends on its strength, the relative position of the animal and the vane, and the sensitivity to which the latter is adjusted, preliminary experiments showed a good overall correspondence between activity observed and that recorded. The pattern of activity was determined by plotting the number of contacts per hour, representing activity, against time as the abscissa.

The experimental tank, 15 × 30 × 30 cm. in size, was filled with water to a depth of 12 cm. It was surrounded with black paper from the bottom to a few centimetres above the water-level. The tank was kept in an easterly window well exposed to daylight and at night to the very much lower light intensities from street lighting and occasional road traffic.

In most experiments the temperature of the water in the tank was recorded continuously by a thermograph. Seven or eight ammocoetes were used in each experiment, of which there were three series. In each series the ammocoetes were placed in the tank for 2–3 days and their activity recorded. In one series enough mud was then added to the tank to cover the bottom to a depth of 2 cm. In the second a number of $5 \times \frac{1}{2}$ in. water-filled test-tubes, corked so that their overall density was just greater than that of the water, were scattered on the bottom. In the third series the tank was covered by a light-proof box. In each series activity was recorded for a further 2–3 days.

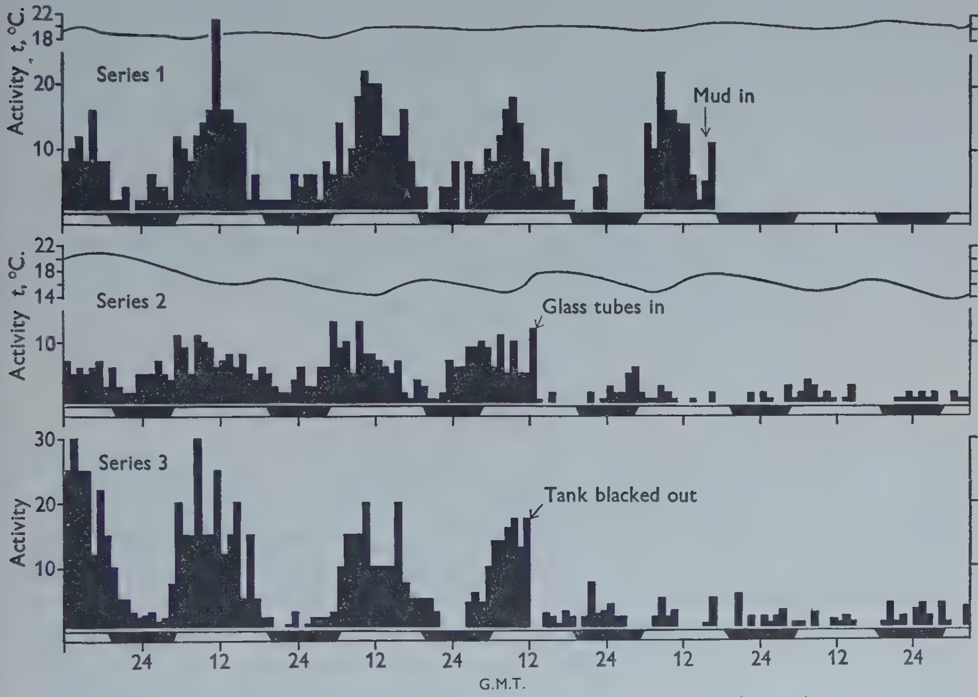

Fig. 3. Aktographs of ammocoetes typical of the three series of experiments.

Results

Results typical for the three series are given in Fig. 3. The ammocoetes were more active during the day than during the night, and the rhythm appears to be independent of temperature fluctuations. Activity usually increased at first light to reach a peak shortly before noon, and then declined to a low level during the afternoon and night. Not all records conformed to this pattern and on two or three nights activity increased long before dawn. Consideration of cloud-cover, age and time of rising of the moon have shown that these disturbances of the normal rhythm cannot have been a lunar effect.

When mud was put into the tank in the first series of experiments there was an immediate increase in activity which was seen to be associated with burrowing. Within an hour all the ammocoetes had burrowed into the mud and only a few tails were left protruding. They did not come out of the mud during the next 40 hr. and the experiment was then stopped. There was a great decrease in overall activity after test-tubes were added to the tank in the second series of experiments. Only weak traces of the rhythm persisted. The larvae could be seen to be lying between the tubes and rarely moved. In the third series, covering the tank with a light-proof box led to the disappearance of the normal rhythm of activity.

Discussion

The results show that there is no inherent rhythm of locomotory activity to parallel that of the melanophores. But movement did not cease in the dark or at night and the decrease in activity which occurred in the experiments with the test-tubes suggest that this residual or basal level is due to a lack of sufficient contact stimulation, to which ammocoetes have been shown to respond by Francis & Horton (1936). Mud, however, appears to give enough contact stimulation to inhibit this activity. The maximum light intensity falling on the water surface was of the order of 1000–2000 m.c. As light passing through 1 cm. of mud was found to be reduced in intensity ten-thousandfold and the threshold level of the ammocoetes lies between 2·5 and 30 m.c. (Francis & Horton, 1936; Stevens, 1950), the mud would also prevent the ammocoetes from being stimulated by light.

ORTHO-KINESIS

Methods

Ammocoetes do not swim continuously even in bright light. As it was not possible to measure the speed at which they moved, an attempt was made to give a quantitative demonstration of ortho-kinesis by recording the time spent on the move at different light intensities. The experiments were carried out in a dark room and the ammocoetes placed singly in a glass tank 20 cm. square and 27 cm. high, filled with water to a depth of 10 cm., and surrounded by a box as shown in Fig. 4. One side of the box was cut away for observation and light entered it through a hole in the roof directly over the tank. The light source for all but the highest intensity was a 200 W. lamp and reductions over 4 log units were obtained using neutral density filters. For the brightest light a 500 W. lamp was used. The light intensities falling on the water surface in the tank were 7000, 540, 54, 5·4, 0·54, and 0·054 m.c. Control observations were made by red light using a Wratten F filter in conjunction with a 1/1000 neutral density filter and the 200 W. lamp. A white background was placed under the tank to make observations possible under red light.

The experimental procedure was as follows. An ammocoete was put in the tank and allowed to settle down in the dark for 45 min. The light was then switched on and observations made for 20 min. The time that the ammocoete spent moving was recorded on a smoked drum by a marker triggered by a hand-operated morse-key.

To avoid disturbance by vibrations, to which ammocoetes appear to be very sensitive, the drum and driving motor were kept on a table apart from the experimental tank. The motor was run continuously and the drum put into gear immediately before observations commenced. In the controls, the red light was left on throughout the settling down period which was of the same duration as in the other experiments. Experiments were made with eleven ammocoetes at each intensity and with twelve under red light.

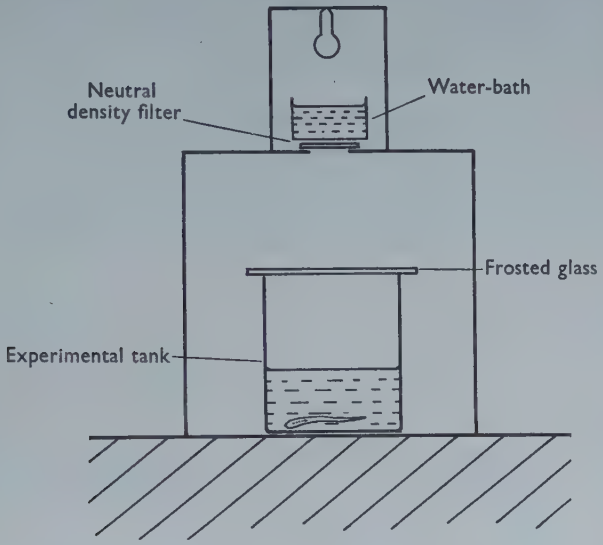

Fig. 4. Apparatus for observing ammocoetes at different light intensities.

Results

The results are given in Table 1 and represented graphically in Fig. 5. Up to a light intensity of 5·4 m.c. the time that the ammocoetes spend moving hardly differs from that observed in the control experiments under red light. At higher intensities the time spent on the move tends to increase linearly with the logarithm of the light intensity.

Discussion

The results show that the time spent moving by ammocoetes is only affected by light intensities greater than 5·4 m.c. and that the threshold for the response lies between 5·4 and 54 m.c. For comparison, the threshold intensity to initiate swimming movements is given as 30 m.c. by Francis & Horton (1935) and between 2·5 and 10 m.c. by Stevens (1950).

The ammocoetes were not at rest at intensities below threshold. In the previous experiments activity was recorded from ammocoetes at night and in the dark. Enequist (1937) states that ammocoetes may come out of the mud at night to swim in the water. Stevens (1950) observed that in nature ammocoetes 'may swim freely in the water at night' and Francis & Horton (1936) found that they 'were very persistent in utilizing the hours of darkness to escape from the glass dishes in which they were confined'. These observations point to the conclusion that ammocoetes do not necessarily remain at rest in the dark, and suggest a possible functional significance of the diurnal rhythm of the melanophores. Obreshkove (1921) found that tadpoles with contracted melanophores had a quicker reaction time to an increase in light intensity than those whose melanophores were expanded. If this was also true of ammocoetes, it might be of advantage to them as it would increase their sensitivity to the first light of day. But there is no evidence to support this suggestion. The melanophores that contribute to the diurnal colour changes lie mostly just below the epidermis (Young, 1935*b*), while the photoreceptors lie within the epidermis (Stevens, 1951).

Table 1. *Time spent moving by ammocoetes during a 20 min. observation period at different light intensities*

Light intensity (m.c.) ...	Red light	0·054	0·54	5·4	54	540	7000
No. of experiments	12	11	11	11	11	11	11
Mean time spent moving (in seconds)	248	246	270	264	347	410	471
Standard deviation	105	86	101	91	138	100	97

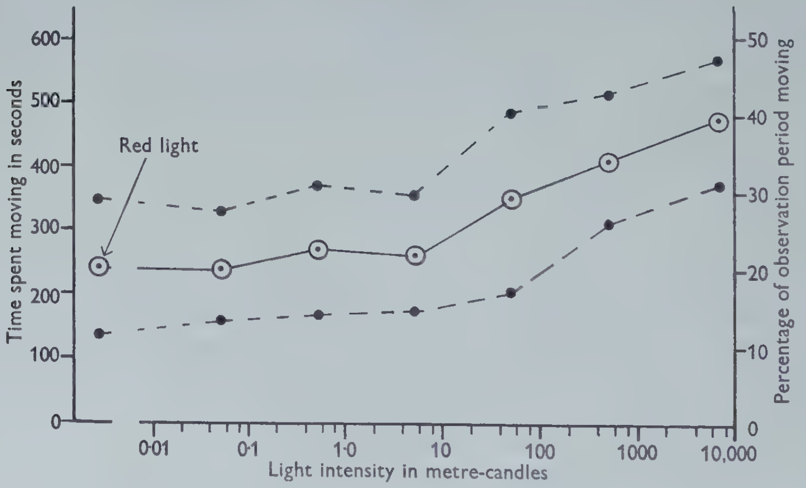

Fig. 5. Time spent moving by ammocoetes at different light intensities.

GRADIENT EXPERIMENTS

Methods

Observations were made on ammocoetes in a horizontal light gradient using a Perspex trough, 4 m. long and 12 cm. square in section, filled with water to a depth of 8 cm. As shown in Fig. 6, the trough was roofed to allow of vertical illumination through frosted glass from one or other of two 100 W. lamps mounted in housings at either end. Black paper vanes hung from the frosted glass to the water surface and round the side of the trough, giving it the appearance of being divided into twenty equal compartments. One side of the tank was backed with black paper while observations were made from the other. To facilitate the identification of a particular compartment, they were grouped together in fours into five sections. All surfaces likely to reflect light were painted matt black.

The construction of the gradient was fundamentally similar to the 'non-directional' gradient described by Ullyott (1936). Non-directional or diffuse light usually means equality of intensity in all directions. Under-water intensity measurements were made of the vertical illumination and of the two longitudinal components of horizontal illumination in different compartments of the trough, which was cleaned and filled with fresh tap water immediately before the measurements were made.

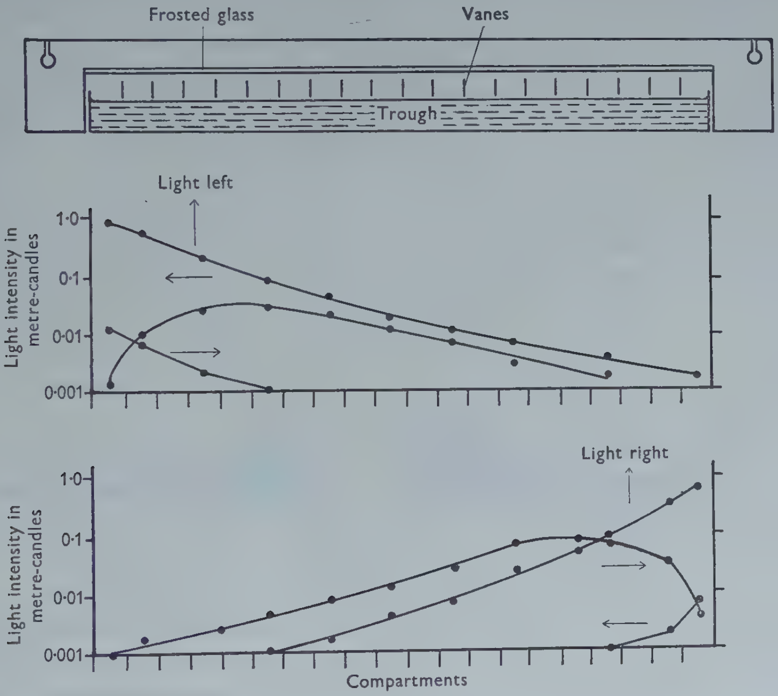

Fig. 6. The vertical and the two horizontal components of light intensity in the gradient. The arrows indicate the direction of the photometer head.

The results are shown in Fig. 6. When the left lamp was on, vertical illumination was always greater than horizontal illumination, and of the two horizontal components, the greatest intensity was recorded when the photometer head was facing the left, that is, towards the brighter end of the gradient. A similar relationship for the horizontal measurements was obtained when using the right lamp. But here the vertical illumination was found to be less than the horizontal in most compartments. No explanation could be found for this anomalous result. However, it provided a control against the distribution obtained with the left lamp.

The measurements show that the gradient has a directional component as the horizontal light is of a greater intensity when the photometer is towards the more highly illuminated end of the gradient than when it faces the dimmer end. The directional component must be partly due to the scattering or reflexion of light by small particles in the water which has been described by Atkins & Poole (1952). As this appears to be unavoidable, it is difficult to see how a non-directional gradient can ever be obtained under laboratory conditions.

Experiments were made with one ammocoete in the gradient at a time. The larva was left in the gradient overnight, in the dark, to settle down. The next morning one of the lamps was switched on and the compartment occupied by the ammocoete recorded at 10 sec. intervals for the next 50–60 min. The lamp was then switched off and after an hour the lighting reversed and a further set of observations made. This procedure served as a control against possible temperature differences in the gradient and against the possibility of the ammocoete establishing a preference for any particular region. The compartment occupied by the ammocoete was recorded in pencil on paper booklets using two colours to indicate whether or not it was moving. After preliminary experiments, a complete series of observations were made with nine different individuals.

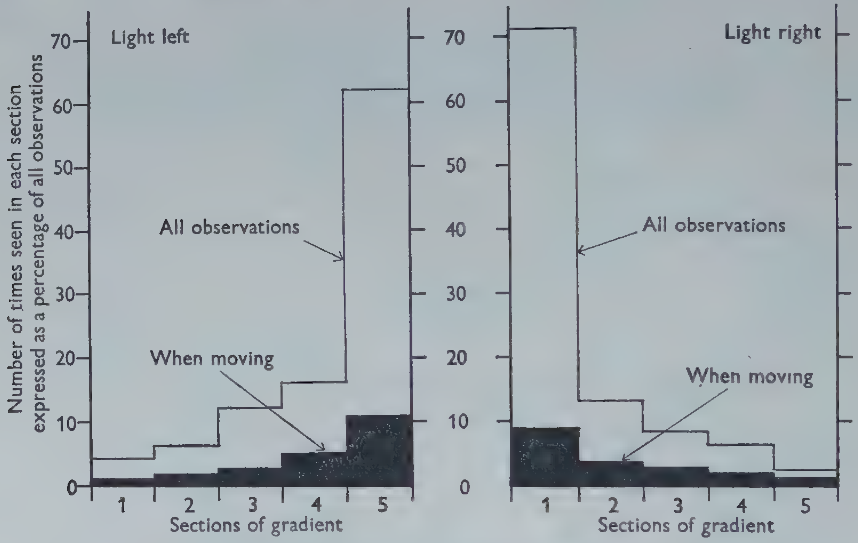

Fig. 7. Distribution of ammocoetes in different sections of the gradient.

Results

The ammocoetes spent most of their time in the darker sections of the gradient and changed ends when the lamps were reversed. The different relative intensities of the horizontal and vertical illumination were without any effect on their distribution. This is clear from Fig. 7, where the number of times that the ammocoetes were observed in each of the five sections of the gradient is expressed as a percentage of the total number of observations made in all the experiments. The data can be divided according as to whether the ammocoetes were moving or at rest. The number of times that they were observed moving in each section is shown in the same figure, and here again the results are expressed as percentages of the total number of all observations. It is evident that the ammocoetes spend both more time at rest and more time moving in the darker parts of the gradient.

Discussion

The maximum light intensity in the gradient was of the order of 1 m.c. which is below the threshold level for ortho-kinesis. Nevertheless, the ammocoetes collected in the dark end of the trough. The results suggest that they avoid the light when swimming and this is the impression gained by observation. When an ammocoete resting in the dark end of the gradient started to swim, it never moved far up the gradient and soon turned back. While it might turn round again and swim towards the brighter end of the trough, it rarely reached it. The ammocoetes appeared to respond to the different intensities or changes in intensity to which they were subjected when moving in the gradient in such a way that they were usually kept in the dark. Although there is a directional horizontal component in the gradient, the reaction can hardly be a taxis as Young (1935*a*) has clearly shown that ammocoetes show no orientation to the direction of the light. The evidence strongly suggests that a klino-kinesis is responsible for the observed aggregation, although a really convincing demonstration of the mechanism could only be obtained by a continuous record of their tracks. But it was not possible to do this.

GENERAL DISCUSSION

The results described in this paper provide a quantitative demonstration that ammocoetes react ortho-kinetically when stimulated by light of intensity above a threshold lying between 5·4 and 54 m.c., and that they probably react klino-kinetically to lower intensities. The suggestion that a moving ammocoete reacts klino-kinetically to intensity changes which are not strong enough to stimulate movement when it is at rest has a parallel in the reactions of fish to temperature. Sullivan (1953) has described how the movements of an active fish may be influenced by temperature changes to which the fish shows no response if it is initially at rest. There appears to be no reason to suppose that the reactions of the ammocoete to light have any directional relation to the source of stimulation, and all the evidence points to the conclusion that their movements are entirely at random. Their behaviour in a gradient or in a light-dark boundary experiment can be adequately described in terms of ortho- or klino-kinesis, or a combination of the two.

There remains the problem of *Proteus*, and the reasons which led Hawes (1945) to conclude that its reactions to light were not truly at random. As he was unable to record tracks of *Proteus* for analysis, another method was devised to test the apparent randomness of its movements. The method is best described in his own words: 'Since in any field illuminated at an intensity provoking photokinesis all movements are at random, it follows that the field of stimulation is uniform throughout in effect; for, if slight variations in any factor (e.g. intensity) were translated into differential activity affecting orientation, the movements would not be at random. Within the limits for photokinesis all light produces the same response, and small variations in the fields will not produce any consistent trend altering the manner of migration from light to dark. The object of the following experiment was to record migrations from light to dark in two types of apparently photokinetic fields differing slightly in the distribution of intensity at the light end, and to compare results. If no consistent differences were found, all movements would be truly random and the conditions for photokinesis fulfilled; if, on the other hand, significant trends were established, they would indicate the existence of determining factors, even if subjective observation could merely disclose apparently random activities.' A stone tank was used in these experiments, half of which was roofed and curtained to the water surface. Illumination was provided by a lamp placed either above the centre of the tank near the curtain, or at the far end of the unshaded half. The time taken for comparable numbers of *Proteus* to move from the light into the shaded half of the tank were recorded under the two conditions of illumination. It was found that the light half was cleared more rapidly when the dark half could be reached by moving down the light gradient than when travelling against it. Hawes concluded that '...although all movements were apparently at random, the alteration in the position of the light source did have a constant effect on the way in which the animals escaped to darkness. The haphazard movement which characterizes photokinesis could not adequately explain this fact.' This conclusion could be correct if the argument was based on sound premises. However, it is not true to say that 'the field of illumination is uniform throughout in effect' or that 'within the limits for photokinesis all light produces the same response'. The nature of the response, that is movement or turning, will certainly be the same throughout the field. But the speed of movement, or frequency of turning will vary with the intensity of stimulation. The idea that the magnitude of the response is dependent on the strength of stimulation is an essential part of the concept of a kinesis as defined by Gunn *et al.* (1937) and Fraenkel & Gunn (1940). A consideration of Ullyott's (1936) results with the planarian *Dendrocoelum lacteum* suggest that it would behave similarly to *Proetus* under the conditions of Hawes's experiments. His results can be adequately explained with the assumption that *Proteus* is responding klino-kinetically to the different intensities to which it is subjected when it moves up or down the light gradient. There appears to be no reason to suppose that the locomotory reactions of *Proteus* to light are any the less at random than those of the ammocoete larva.

SUMMARY

1. Ammocoetes show no inherent rhythm of locomotory activity to parallel that demonstrated for their melanophores.

2. They react ortho-kinetically to light above a threshold lying between 5·4 and 54 m.c.

3. Ammocoetes collect in the dark end of a light gradient at intensities below the threshold for ortho-kinesis. A klino-kinesis would appear to be the mechanism by which this aggregation is accomplished.

4. All the evidence points to the conclusion that the locomotory reactions of the ammocoete to light bears no directional relationship to the source of stimulation and that they are entirely at random.

These experiments were carried out at the Zoological Laboratory, Cambridge, and I am very grateful to Prof. Sir James Gray, F.R.S., for letting me work in his Department. I wish to thank Dr R. H. J. Brown and Mr J. A. Popple for their help in connexion with electrical apparatus, and Dr H. W. Lissmann, F.R.S., for his interest in the work. While part of the work was carried out I was in receipt of a grant from the Development Commission.

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COMPARATIVE STUDIES OF THE THYROID FUNCTION IN TELEOSTS OF TROPICAL AND TEMPERATE HABITATS

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The presence of thyroid gland tissue in teleosts was first reported by Simon (1844), and later its structure was shown to consist of follicular units similar to those in the thyroid gland of higher vertebrates. The teleostean gland is not discrete but consists of numerous scattered follicles.

Marine & Lenhart (1910) observed a hyperplastic condition of the thyroid tissue in some trout in hatcheries which was correlated with sluggishness, altered pigmentation and fatness. This condition suggested an altered metabolism. Feeding experiments with mammalian thyroid tissue (Etkin, Root & Mofshin, 1940; Smith & Everett, 1943; Hasler & Meyer, 1942; Root & Etkin, 1937) failed to alter oxygen consumption of teleosts. Only a few individuals showed a rise in oxygen consumption when fed teleostean thyroid tissue (Smith & Matthews, 1947). Thyroid hormone production in mammals is depressed by thiourea. Matthews & Smith (1947) however, were unable to demonstrate a depression of oxygen consumption in *Fundulus* by the use of this drug.

As growth and development of fish may be determined to some extent by the presence of thyroid hormone, experiments to elucidate this effect have been carried out. Some observers have reported a positive effect on giving thyroid hormone (Grobstein & Bellamy, 1939; Goldsmith, Nigrelli, Gordon, Charipper & Gordon, 1944; Hopper, 1952; Vivien & Gaiser, 1952), and others (Smith & Everett, 1943) the reverse. Using thiourea, Smith, Sladek & Kellner (1953) recorded a depressive effect on the growth of *Lebistes*, but they attributed this to a slight toxic effect on the drug.

These contradictory results prompted the work reported below. If the thyroid mechanism regulates metabolism in the teleostean fish, it might be expected that a study of such fish at two temperature extremes would throw some light on the problem. A comparison of the effects of temperature change on thyroid activity, as exemplified by structural changes of the thyroid, and on growth and development afforded a promising approach. Fish from temperate and tropical climes were selected and these were studied under different environmental temperature conditions. As seasonal variations in the thyroid gland of many teleosts are known to occur it was necessary to extend observations over a whole year (Lieber, 1936; Hoar, 1939; Buchmann, 1940).

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MATERIAL AND METHODS

Two species of fish were selected; the temperate form *Phoxinus laevis* (*Phoxinus phoxinus*) and the tropical form *Lebistes reticulatus*. In both, the thyroid gland consisted of typical follicles scattered along the ventral aorta with occasional invasion into the adjacent muscle bands.

Phoxinus laevis, rarely longer than 6 cm. is found in lakes and quiet streams in England. It can tolerate a temperature range from that of ice-covered water to 23° C. though it has been taken from water at a temperature of 31° C. on the continent of Europe. Spawning occurs from May to July (Frost, 1943). Supplies were obtained from Lake Windermere except during January 1952 when they came from Surrey as the fish retire to deep water in Lake Windermere during the winter.

The fish were kept in large glass jars containing 2 l. of well-aerated tap water or in solutions made up with tap water. The jars were immersed in water-baths whose temperature could be controlled to ± 1° C. The fish were fed with enchytraeid worms and horse flesh twice weekly and the solutions changed after feeding. Mortality was negligible.

Lebistes reticulatus is a small tropical teleost living within a temperature range of 15–35° C. with an optimum of 25° C. The females are viviparous, and are capable of producing a brood every month. As the 'pregnancies' affect the thyroid gland structure (Stolk, 1951) the fish, which were bred locally, were chosen so that each experimental batch contained both young fry and older fish. The fish were kept in a litre of tap water or solution made up with tap water. The tap water was allowed to stand for at least a week before use so as to reduce the amount of chlorine present. The glass jars containing the fish were kept in water-baths at a constant temperature. Aeration was not necessary. Feeding was daily and solutions were changed twice a week. Some deaths inevitably resulted from the change.

The most suitable concentration of thiourea used as an anti-thyroid drug was determined during a preliminary study on *Lebistes*. It was found that a concentration of 0·05 g. thiourea per 100 ml. water was the most concentrated solution which caused no deaths during 7 days.

The temperature levels used were: *Phoxinus*—3°, 15, 20 and 25° C.; *Lebistes*—20, 25 and 30° C. Twenty fish were used for each experiment, ten in thiourea solution and ten in tap water as controls at each temperature level. *Phoxinus* controls could not be kept alive at 25° C. One fish was killed from each group when the water was changed twice weekly. Histological examination of the thyroid gland was carried out on each specimen. Fixation was in Bouin's fluid. Sections were stained with Masson's trichrome stain.

The measurement of thyroid activity. An estimate of the degree of thyroid activity is based frequently on the structural changes found in the gland such as: (*a*) alterations in the height of the follicular epithelial cells; (*b*) changes in the amount of colloid found in the vesicles; (*c*) the altered position of the nucleus of the follicular cell. The changes produced in the mammalian thyroid gland by the administration of thyrotrophic hormone (TSH), which stimulates production of thyroid hormone (TH), are identical with those found when anti-thyroid drugs such as thiourea are given. The latter drug inhibits the production of TH and consequently the concentration of TH in the blood falls which in turn is the adequate stimulus for increased production of TSH. Thus the morphological signs of increased thyroid activity are not necessarily related to the rate of TH production. In fact, the degree of stimulation of the thyroid gland is demonstrated by these signs, the extent of morphological change depending upon the relative levels of TSH and TH. If the thyroid gland is producing large quantities of TH, inhibition of this production will cause a change in the TSH:TH ratio with a consequent marked and rapid change in gland structure. If the gland is producing little TH, inhibition of production of TH will cause only a small disturbance in the TSH:TH ratio, hence the resultant stimulation of TSH production will be small and structural changes will not be marked. The apparent changes in thyroid activity seen in histological material can thus be used as indices of an alteration in the TSH:TH ratio. Various methods have been devised to measure the degree of activity manifested by the thyroid gland in response to TSH. The most general is to measure the height of the follicular epithelium. Cell height increases with a rise in the rate of production of TSH. This method, however, is only reliable when under control conditions, the follicular cells are reasonably even in height. In teleostean fish, cell height is very variable even in the same follicle. Small follicles have a lower epithelium than large follicles. When activity increases the fish thyroid tends to show proliferation of the follicular epithelium with consequent formation of new follicles. The method of measuring thyroid activity suggested by Uhlenhuth *et al.* (1945), and used in a study of the salamander thyroid gland, was adopted. This consists of determining on sections of the thyroid gland the relative areas of the follicular epithelium and follicular colloid. In the two fish species used here the amount of colloid present was inversely proportional to the height of the follicular epithelium. Stained slides were projected on to squared paper. The outlines of the outside of the follicles were traced and also the outlines of the inner edge of the epithelium. The area represented by the follicles was cut off and the paper weighed. The area representing the cells was then cut off and weighed. The ratio of 'cell weight' to 'total follicular weight' was then expressed as a percentage, indicating thyroid activity. It was found that when the ratio, calculated from the actual areas of the follicles and follicular cells, was compared with that determined by the weighing technique there was an agreement within ± 1 %. The mean ratio from twenty-five follicles was determined for each specimen.

The advantages of this method of assessing the state of activity of the thyroid gland are: (1) irregularities of the follicles and cell proliferations are taken into account; (2) the amount of colloid present is also taken into account in the ratio; (3) the value of the ratio differs less between large and small follicles than the difference in activity as estimated by differences in cell height in follicles of different sizes.

The chief disadvantage of this method is that there is a lower limit below which measurement is inaccurate. In very small follicles there is relatively little colloid and hence the ratio appears high. This however is cancelled out by measuring a large number of large and small follicles. It is clear that the ratio can be disturbed by two factors: (*a*) changes in the height of the epithelium, and (*b*) changes in the amount of colloid formed. The ratio, although a purely arbitrary method of assessing the level of thyroid activity, certainly will show direction of change even if the amount shown by variations in the ratio may not be an exact reflexion of the altering levels of thyroid activity. This method also enables the average size of the follicles to be determined, the number of follicles less than a certain arbitrary size to be estimated and the percentage of different staining colloids within the follicles to be determined.

EXPERIMENTAL

Effect of changes of environmental temperature on thyroid activity

Phoxinus

The experiments described above were carried out over a period of 5 weeks, and repeated at intervals throughout the year. The experiments were divided into two groups: (*a*) controls, (*b*) fish exposed to thiourea. Environmental temperatures used were: 3, 15, 20° C. in the control group; 3, 15, 20 and 25° C. in the thiourea group.

The results found in the period April to May are given in Table 1 and Fig. 1 A, B. This period may be regarded as representative. The mean values of the control group

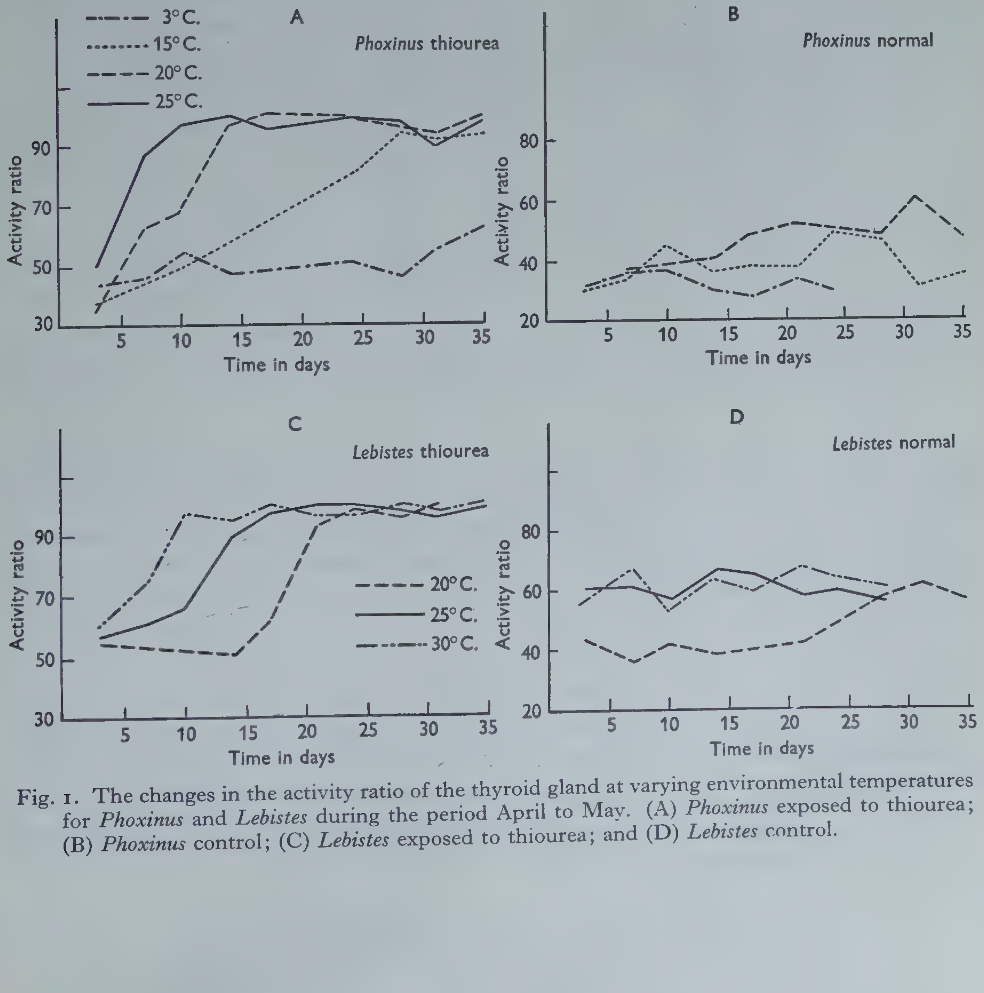

Fig. 1. The changes in the activity ratio of the thyroid gland at varying environmental temperatures for *Phoxinus* and *Lebistes* during the period April to May. (A) *Phoxinus* exposed to thiourea; (B) *Phoxinus* control; (C) *Lebistes* exposed to thiourea; and (D) *Lebistes* control.

Table 1. *Effect of temperature on the thyroid activity of teleosts*

Phoxinus. April to May. Activity ratio

Days	25° C. Thiourea	20° C.		15° C.		3° C.	
		Thiourea	Control	Thiourea	Control	Thiourea	Control
3	50·0	36·5	50·0	36·8	30·0	43·8	31·4
7	87·5	62·5	36·1	58·3	34·3	46·6	36·1
10	96·5	68·7	23·7	50·0	45·0	55·5	37·4
14	99·7	97·5	40·9	69·2	36·5	48·0	30·3
17	96·1	100·0	48·2	44·0	38·2	49·7	27·3
21	75·0*	82·9*	52·3	54·0	38·0	36·1	34·1
24	100·0	100·0	50·0	80·6	50·0	52·1	30·0
28	98·2	96·1	49·3	95·7	48·3	47·4	—
31	90·7	94·3	62·5	93·2	32·6	56·7	—
35	98·0	100·0	47·4	94·6	35·6	63·9	—
Mean	—	—	46·0	—	38·9	—	32·4
S.D. ±	—	—	9·9	—	6·5	—	3·4
S.E. ±	—	—	3·2	—	2·1	—	1·2

Lebistes. April to May. Activity ratio

Days	30° C.		25° C.		20° C.	
	Thiourea	Control	Thiourea	Control	Thiourea	Control
3	61·0	55·5	57·1	60·0	55·5	44·6
7	75·1	66·6	62·3	61·6	50·0	37·3
10	98·0	53·8	67·2	56·5	62·3	43·4
14	95·7	63·6	91·7	68·7	50·0	39·2
17	100·0	59·7	98·2	65·5	62·2	40·5
21	96·5	67·8	100·0	58·3	94·4	42·8
24	96·8	62·4	100·0	60·1	99·7	60·0
28	100·0	60·0	98·7	57·9	96·4	57·6
31	98·0	—	96·3	—	100·0	62·3
35	100·0	—	99·2	—	—	55·5
Mean	—	61·2	—	61·1	—	48·3
S.D. ±	—	4·6	—	3·9	—	8·9
S.E. ±	—	1·6	—	1·4	—	2·9

S.D. = standard deviation; S.E. = standard error of the mean.
* Show an unusual lack of reaction.

show clearly that thyroid activity does not increase as the environmental temperature is lowered. It will be noted in the thiourea group that at 3° C. the highest level of activity (63·9) was attained only after 5 weeks. At 15° C. a 95·7 activity ratio was attained on the 28th day. A level of activity of 90 was reached at 20° C. between the 10th and 14th day. At 25° C. this same level was reached between the 7th and 10th day.

Lebistes

Similarly the experiments were divided into two groups: (*a*) control, (*b*) exposed to thiourea. Environmental temperatures used were: 20, 25 and 30° C.

The results found in a representative period (April–May) are given in Table 1 and Fig. 1, C, D. The results again indicate that thyroid activity increases with increased environmental temperature. The mean value of the control groups (61·2 activity ratio at 30° C., 61·1 at 25° C., 48·3 at 20° C.) demonstrates this. The thiourea-treated groups behaved in a like manner; the fish at 30° C. taking between 7 and 10 days to reach a thyroid activity ratio of 90, between 10 and 14 days at 25° C. and between 17 and 21 at 20° C., to reach the same activity ratio.

It has already been noted that *Phoxinus* could not be kept alive at 25° C., unless treated with thiourea. No attempt at gradual acclimatization to this increased temperature was made, but when treated with thiourea the fish survived indefinitely and appeared normal. Hence thiourea treatment must affect the thermal death-point. To test this *Phoxinus*, already treated with thiourea for 3 days, were subjected to a rise in temperature of 10° C. (i.e. to 33° C.) over 2 days. They survived indefinitely at this increased temperature and appeared normal, the controls dying under such conditions between 23 and 24° C.

Lebistes has a higher thermal range but this could also be extended by thiourea treatment.

The thermal level of response to thiourea is higher in *Lebistes* (20° C.) than in *Phoxinus* (3° C.).

Seasonal changes in thyroid activity

These experiments were designed to determine if the possible seasonal variation in thyroid activity was related to the external temperature to which the fish was subjected, or if there was an inherent rhythm associated with the breeding cycle. *Phoxinus* has a yearly breeding cycle which could be related to annual variations in thyroid activity. *Lebistes*, on the other hand, breeds monthly throughout the year.

As each of the above experiments was repeated at intervals throughout the year, similarly treated specimens could be compared for any 2-monthly period. A complete record was obtained except for the month of March.

The results of the control experiments with *Phoxinus* showed that there were two peaks in thyroid activity, one during April-May and the other during late August-September which were common to all three environmental temperatures (Fig. 2A). *Lebistes* showed no distinct variation common to all temperatures (Fig. 2B).

Effect of thiourea on growth

Phoxinus. Eggs were obtained from Lake Windermere in May. A similar technique was again used and the experiments were divided into two groups: (*a*) control, (*b*) immersed in 0·05% thiourea. There were approximately 600 specimens in each. Half the volume of solution was changed thrice weekly. The eggs were placed in the fluid in shallow glass troughs and aerated vigorously. The majority hatched out after 3 days. Three days later feeding was commenced. Protozoa culture was given three times a day for the first month, with small amounts of a dried fry food and living *Daphnia* at the end of the third week; the Protozoa culture was then discontinued and the other food was given twice daily. Specimens were fixed at first each day, with gradually lengthening intervals as the fry became larger. Very small specimens required double embedding.

The results show that the gland develops as a solid mass of tissue from the ventral region of the pharynx between the first and second gill slits. After 13 days, the control group show a few follicles with low epithelium and abundant green-staining colloid. Those treated with thiourea have a high epithelium, small amounts red-staining colloid and an increased number of follicles. However, although thiourea treatment produces marked changes in the thyroid structure, growth of the fish is not permanently affected. There was a slight difference in the average size of the fish in the two groups between the 15 and 35 days after hatching (thiourea, average length 8·5 mm., control, 9·5 mm.). This was only temporary and the average length became the same in both groups and reached 11 mm. 3 months after hatching. The thiourea-treated fish still showed marked thyroid hyperplasia.

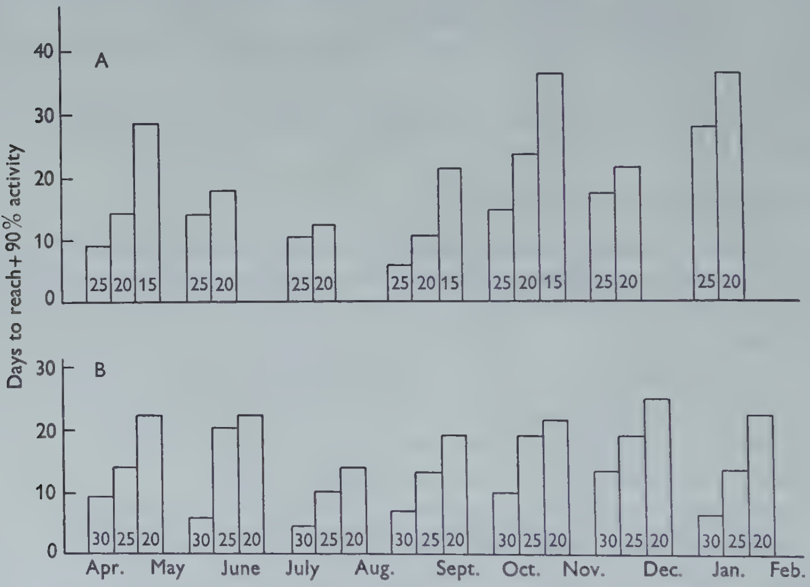

Fig. 2. The time taken for the thyroid gland to reach 90 % or more activity, under thiourea treatment, at different times of the year. The varying speeds are indicative of variations in thyroid activity. In *Phoxinus* (A) the variation is seasonal, the thyroid being less active during the winter. Only during the more active periods do the values at 15° C. rise to 90 %. *Lebistes* (B) shows much less variation, which is random. The figure inscribed in each rectangle gives the temperature in degrees centigrade.

Lebistes. A similar technique was used, but as *Lebistes* is viviparous a third group was included: (*a*) control; (*b*) thiourea-treated from birth; (*c*) fry from a pregnant female previously kept in thiourea for 3 weeks before birth, the treatment of the fry being continued afterwards.

The fry may have a yolk-sac at birth or if more completely developed, may be without one. Any differences were noted. Specimens were killed daily and treated as before for histological examinations.

There was no size difference between fry treated with thiourea for 4 weeks and normal fry of the same age (average length 6·5 mm. at birth, 8·5 mm. at

4 weeks). There was a differentiated thyroid in all fry at birth, with the control fish having well-developed follicles, low epithelium cells and abundant colloid. All thiourea-treated fry showed a hyperplastic thyroid and colloid absent or reduced.

DISCUSSION

The modern conception of the action of anti-thyroid drugs is based almost entirely on work with avian and mammalian thyroids. It is generally accepted that there is a partial or complete blockage of thyroid hormone synthesis. Histological changes take place in the teleostean gland, after thiourea treatment, which are analogous to those occurring in mammals and birds after similar treatment. The experiments outlined here show that thiourea treatment causes a change in the temperature range of the two species of teleosts used. This is a definite physiological effect, and justifies the conclusion that thyroid hormone production has been reduced by this method.

When the fish are placed in thiourea solution a definite time interval is required for the drug to exert its maximum effect at any given temperature. The time taken to reach maximum response to thiourea diminishes with an increase in environmental temperature. Although some glands give ratios of 100, which means that the area occupied by the follicular cells is equal to the total area of the follicle, it is preferable to accept a ratio of 90 as indicating an arbitrary 'maximum' level of activity. The time taken to reach this level of activity can be determined for each temperature level and for each month of the year at the same level. When this is done for a specific monthly period (e.g. April to May) the results show that there is strikingly little variation in the ratio of the total follicular area to cell area in the control groups of both species at any given temperature, over a period of 3–5 weeks. On the other hand, the ratio for the thiourea-treated fish gradually increases over the same time interval (see Table 1 and Fig. 1). The mean control values indicate a depression of thyroid activity with lowered environmental temperatures. This is emphasized by the varying speed of reaction to thiourea which is more rapid at high temperatures than at low. If cold, as well as thiourea, had stimulated the gland the reverse would have been found. These results therefore show clearly that the thyroid gland is stimulated to increased activity on exposure to higher environmental temperatures. Obviously these results are the reverse of those to be expected were the thyroid gland acting to maintain some uniformity of heat production.

The arbitrary standard set up allows a comparison between states of activity at different times of the year. If a true seasonal variation occurs, it should be apparent at all those levels of environmental temperature at which the thyroid has been shown to be reactive. It is shown in Table 1 that, with the exception of *Phoxinus* at an environmental temperature of 3° C., such a reactive condition obtains. *Phoxinus* shows a seasonal variation during thiourea treatment at 15, 20 and 25° C. At 3 and 15° C., although a ratio of 90 is not attained during most months, a higher level of activity is reached at the spring and summer peaks. The peak activity is reached in early spring (April to May) and late summer (August to September). During the winter the lowest activity is found from December to February. Bullough (1939) has shown that there is an increase in gonad size in *Phoxinus* twice during the year; September to October and in the spring. The thyroid activity peaks precede the increase in gonad size, and it may be assumed that there is some relationship between the two.

Lebistes shows an indefinite variation. Although a peak activity is apparent at 20° C. during July to August it is not present at the other environmental temperatures. It is therefore not a true seasonal peak which would be present at all environmental temperatures. As the breeding cycle is monthly it appears that seasonal variation is correlated with an annual breeding cycle.

No effect of thiourea treatment on growth could be demonstrated.

SUMMARY

1. Normal and thiourea-treated *Phoxinus* and *Lebistes* show a depression of thyroid activity by low environmental temperatures and stimulation by high.

2. Thiourea treatment affects the thermal range in both species.

3. *Phoxinus* shows a seasonal variation in thyroid activity, correlated with breeding periods. This is absent in *Lebistes*.

4. No effect of the thyroid on growth could be demonstrated.

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WAX SECRETION IN THE COCKROACH

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The production of massive quantities of waxy materials by bees and similar insects has been known for a very long time, but it was not until 1938 that Bergmann showed the existence of smaller quantities of wax in the cuticle of *Bombyx*. Ramsay (1935) was the first to demonstrate that a greasy material on the surface of the cuticle was responsible for waterproofing the cockroach, and subsequently the work of Wigglesworth (1945) and Beament (1945) produced evidence for believing that the existence of a thin layer of wax in the epicuticle was widespread throughout the insects, and was the chief mechanism for restricting water-loss. It is now known that wax layers are also present in ticks (Lees, 1947) and in many arthropod egg-shells (Beament, 1946, 1951, etc.), while Chibnall, Piper, Pollard, Williams & Sahai (1934) have carried out a detailed analysis of beeswax and of the wax of the white pine chermes. These waxes consist of long chain alcohols, paraffins and acids; the aliphatic chains always appear to be saturated. The similarity of the physico-chemical behaviour of cuticular waxes with beeswax, as outlined by Beament (1945), seems to leave little doubt that the minute but essential amounts of wax in typical insect cuticle are composed of the same type of chemical components as those found in beeswax. The range of melting-points of these materials shows that relative changes in the chain length of the component would be sufficient to account for the differences between the specific properties of waxes derived from different species.

THE SECRETION OF WAX

Wigglesworth's extensive work in 1947 and 1948 on the structure and formation of the cuticle has shown a sequence of processes by which an epicuticle is formed. In *Rhodinius*, it seems that during the deposition of the cuticle, a wax appears over the surface of a semi-liquid protein layer rich in polyphenols which will subsequently become the 'polyphenol layer' of the epicuticle. At this stage the 'wax' can readily be removed, but later it has become a harder material. In the waterproofing of eggs by the tick (Lees & Beament, 1948) it is clear that the wax, rendered water-dispersable in the reservoir of Géné's organ by being linked with protein, is a mobile material completely devoid of protein by the time it is produced at the surface of the organ. This material will spread over the surface of a freshly produced egg, and for a short time afterwards it is sufficiently mobile to pass from one newly laid egg to another which has not been in contact with the organ. But it soon becomes a hard, non-spreading material, and it is important to note that when the organ is made to secrete, but with no eggs to receive its secretion, wax accumulates on its surface with much inferior spreading properties.

Even more remarkable in this respect is the plastic waxy substance with which the red spider mite *Metatetranychus* coats its egg (Beament, 1951). This can be shaped into the long spike which surmounts the egg, by the soft ovipositor of the female, as it is withdrawn. Yet after an hour, the extremely brittle wax has a high melting-point, and it is not long before it melts at 170° C. These are outstanding examples of the type of change which is believed to go on in insect waxes immediately following their secretion.

INITIAL EXPERIMENTS

The starting-point of this investigation was a chance observation by Prof. V. B. Wigglesworth (to whom I wish to express my thanks). Grease obtained by washing the external surface of many cockroaches (*Periplaneta*), was stored in a cupboard for several months. Now in its natural state on the animal, this grease is a vaseline-like substance which has no true melting-point; it ceases to be a waterproofing material at about 33° C. But the stored sample had changed to a solid hard material, looking very like a white wax. It had a melting-point of the order of 56° C., it was readily soluble in chloroform, benzene and similar solvents, and its general properties were very similar to those of, for example, the cuticular waterproofing wax of *Rhodnius* (Beament, 1945). When this material was deposited from chloroform on to a membrane of lipoid free locust wing in a membrane holder, it produced reasonable waterproofing, with a transition temperature of the order of 50° C. Generally speaking, therefore, it appeared to be a wax composed of long-chain paraffinic components. Now paraffins, and their related alcohols and acids, are known to be chemically extremely inert materials. Nevertheless, it seemed very necessary to examine all the possible processes by which a change such as this could come about. It is possible that slow chemical change, such as oxidation or reduction, could have altered the materials present; polymerization of shorter components might have produced a wax of larger molecular weight, and therefore higher melting-point: or some enzymic, or even bacterial, process may have gone on.

Accordingly, small samples of grease were obtained by washing living late nymphal cockroaches with cold chloroform, and allowing the solvent to evaporate for 24 hr. Chloroform is readily detected by its smell, and this was used as a crude but probably quite sensitive test for the freedom of the sample from solvent. (It is later shown that these samples might have contained traces of chloroform, but it is most unlikely that this would in any way affect the validity of the results, for one does not expect chloroform to combine with these natural greases, or cause them to change.) These grease samples were then placed in small sealed glass tubes, in atmospheres of nitrogen, hydrogen, oxygen and argon; some were stored in darkness and others in sunlight and, in some tubes, the volume of gas was very small. They were left for periods of several weeks before being opened and examined.

In no case did any hard wax form in these tubes. To check that the process of change had not proceeded at least partially, it would be necessary to determine the melting-point of the samples, but for such a grease this is not possible. It is known (Beament, 1945) that the transition temperature of the grease sample would be a more reliable indication of changes, but again, there is appreciable wastage in making up standard membranes, the amounts of material to be tested were very small, and some micro-method was sought. Now it is known (Ramsay, 1935) that a droplet of water, placed on the cockroach, is rapidly waterproofed by a film of the grease spreading over it. These circumstances cannot be reproduced on clean glass cover-slips which had been coated with grease by being wiped over the surface of a living cockroach; a water droplet, added to the cover-slip, was apparently covered by grease, but very soon made contact with the underlying glass, whence it spread rapidly through the glass-grease interface to the edge of the cover-slip and there evaporated. One needs a surface with different wetting properties.

Now if the glass is replaced by polythene, or polytetrafluorethylene sheet, with which water has a contact angle of 90°, waterproofing can be obtained, and a drop so covered will remain for a very long time at room temperature. If these plastic sheets are then sealed on to the end of a wide glass tube, the other end of which is open, and then suspended vertically so that the sheet is in the surface of a water-bath, the film can be heated slowly, while at the same time preventing its being exposed to the almost saturated air above the bath.The temperature must be raised slowly, for such plastics are very poor conductors of heat, but at the transition temperature, as shown by samples removed directly from living animals, droplets of 1 mm.^{3} disappear in about a minute. This gives a rapid and reliable method for determining transition temperatures for very small samples of grease, and the material from the sealed tubes was so tested. The results are shown in Table 1; this also includes comparative values for a few samples which were large enough to be checked by standard membrane methods. However, determination by membrane holder takes a number of days to perform, and causes the grease to be exposed to

Table 1. *The temperatures at which 1 m.^{3} droplets of water on polythene sheeting evaporate, after covering with various grease films; transition temperatures by membrane holders are given for comparison*

Treatment	Transition temp. by droplet (° C.)	Transition temp. by membrane (° C.)
Living roach	34	33
O_2 dark 3 months	35	—
O_2 light 3 months	38	—
H_2 dark 3 months	35	—
H_2 light 3 months	37·5	—
N_2 dark 3 months	38	37
N_2 light 3 months	35	—
Ar small volume	36	—
Ar large volume	41	42
Rapid heating 55° C.	—	49, 51
High vacuum, room temperature 2 weeks	41(?)	44

higher temperatures for considerable periods; it is possible, in fact, that these values are not as reliable as those obtained from the droplets.

It will be seen from the table that some small changes have taken place in the transition temperatures of the grease samples in the sealed tubes, but none of them have even approached the figure of 50° C. although they have been stored for a period during which complete transformation to hard wax would occur in a freely exposed sample. From our original hypothesis we can certainly eliminate the possibility that direct oxidation is responsible. It also seems most unlikely that the mere operation of sealing the grease in a tube would have prevented polymerization, where every precaution was taken to avoid heating the material; equally well, reduction processes would not have been eliminated, and as no antiseptic precautions were taken, it is hardly likely that bacterial action would have been prevented in this way.

The only evidence of any possible significance is that the samples with the highest transition temperatures come from tubes of greatest enclosed gas space. Is it possible that evaporation is taking place? A sample was heated on a glass slide to 100° C.; the grease was rapidly transformed into one with a higher transition temperature. It was soon discovered that mere prolonged heating would produce a material which would not spread over water at all at room temperature. Subsequently, a large sample of grease kept for several hours on a glass plate at 60° C., changed to a 'wax' with a melting-point between 50 and 55° C. If a sample was rapidly heated to a much higher temperature (bearing in mind the possibility of structural change or decomposition which this treatment might induce) a vapour could sometimes be detected which carried the distinctive odour of cockroaches, but which was not so pungent as to give the impression that it was composed entirely, or even mainly, of this aromatic material; equally, it was not chloroform. In further tests, grease samples were stored at room temperature under high vacuum, and again small but significant increases in the transition temperature were noted; a wax with a melting-point of 50° C. could be obtained in this way much more rapidly than by exposure at the same temperature to the atmosphere.

EVIDENCE FOR VOLATILE COMPONENTS

As a result of these preliminary experiments, it seems that the transformation of grease into wax is not—or is not chiefly—due to a progressive chemical process. Since the changes can be speeded either by heat or by evacuation, there is every reason for supposing that some material is 'lost' in the hardening process. One must presume that the loss of a volatile component of the original grease is the principle cause for the change, and that its evaporation at room temperatures is very slow indeed. Despite the diversity of products secreted by insects, it would be very surprising if they could produce any of the typical wax solvents used in the laboratory, such as chloroform, ether, benzene, etc., which are either anaesthetic or highly toxic substances to them. All one can reasonably assert here, is that such a volatile solvent would have to be a liquid at room temperature, and must therefore be of a low order of molecular weight, especially as compared with wax molecules whose carbon chain may be 24–32 atoms long. The determination of molecular weights of solvent and wax is therefore a valuable approach.

MOLECULAR WEIGHT BY VAPOUR DENSITY

(1) *Vapour density*

It seems likely, from the evidence given above, that most of the volatile materials present would vaporize in a vacuum at temperatures above 60° C. A sample of grease, obtained from chloroform washings of living animals, was allowed to evaporate until no smell of chloroform could be detected. It was divided into two roughly equal parts, one of which was weighed and then heated under reduced pressure at 64° C., thus giving an indication of the weight of volatile material present in the sample. The other portion was weighed and injected into the Torricellian vacuum of a mercury barometer, jacketed with methyl alcohol vapour at 64° C. From the volume occupied by the volatile part of this sample, its vapour density could be calculated: its mean molecular weight is of course twice this figure.

The results showed considerable variation; values for molecular weight varying from 100 to 200 were obtained. It is, however, obvious that if the sample is stored for a long time, solvent and chloroform are both lost, whereas the determination of molecular weight on a fresh sample must include some chloroform vapour. But even if one cannot make a direct suggestion as to the nature of the solvent, these experiments do at least provide concrete evidence for the presence of a volatile component in the grease. Clearly a different method for determining molecular weights is needed.

(2) *Cryoscopic method*

Molecular weights of materials in solution can be obtained by their depression of the freezing-point of a suitable solvent. Ramsay (1949) has described a method for the determination of very small samples of aqueous materials, and his method was adapted for this purpose. Benzene was chosen as the solvent; it has a very large cryoscopic constant as compared with water, and is a good wax solvent. In Ramsay's method the sample is stored under liquid paraffin, and a small sample drawn into a fine silica tube, enclosed in the paraffin. For the present purpose, a liquid insoluble in benzene is needed, and pure glycerine was used. Since benzene is appreciably less dense than glycerine, the samples were stored by injecting them into the highest point of an inverted U-tube of fairly narrow bore filled with glycerine, so that the liquids remained in by capillarity. A number of large nymphal or adult cockroaches were rapidly washed with recrystallized benzene to obtain the grease solution; this was carefully decanted from the collecting dish, for small amounts of watery materials—probably excreta—are often also washed off. The volume was carefully measured and half of the material was evaporated to dryness and heated at 60° C. to drive off the benzene and the solvent; samples of the other portion were stored under glycerine in U-tubes. The dried portion was weighed, taken up in a small quantity of benzene, its volume measured and similarly stored under glycerine. Samples were then drawn from either tube, and their freezing-points determined by Ramsay's method. The freezing-point of pure recrystallized benzene, after storage in glycerine, was also taken, which by subtraction gives the depression caused by the wax or grease in solution. It is appreciated that any inter-solubility between benzene and glycerine might have an effect on the results obtained, but the values for pure benzene after storage in glycerine are very close to those quoted by the manufacturers, using a standard salt solution for calibration of the Beckman thermometer. Certainly, the concentrations of waxes used cause a large depression in freezing-point, as compared with the possible variation in the 'pure' benzene through dissolved glycerine.

From the depression of freezing-point of the sample containing the hard, non-volatile wax component, its mean molecular weight was obtained. The other sample gives the freezing-point of a solution containing a known molecular amount of wax, together with solvent molecules. From this, one can calculate the mean molecular weight of the solvent, for various proportions of solvent to wax. The results are shown in Table 2. On p. 522, the amounts of volatile component present are given, and it is now possible to indicate the range of molecular weight possible for the volatile material; it must lie between 140 and 190. Estimates are also given for the molecular weight of beeswax by this method, and it will be seen that they agree very well with the results published by Chibnall *et al.* (1934).

Table 2. *The molecular weights of various waxes by the depression of the freezing-point of benzene*

$$\left(\text{M.W.} = \frac{\text{g. wax}}{\text{g. benzene}} \times \frac{4900}{\text{depression freezing-poiot}}\right).$$

Material	% volatile material	Molecular weight from freezing-point	Molecular weight of volatile components	Equivalent carbon chain
Hard cockroach wax (see p. 522)	—	420–255	—	C 27–C 29
Stearic acid (M.W. 284)	—	265–280	—	C 18
Beeswax	—	360–390	—	C 25–C 27
Vaporized grease	—	295–350	—	C 22–C 25
Whole grease				
Sample A	41	315	139	C 10
B	37	345	184	C 13
C	29	355	148	C 11
D	27	370	176	C 12

INFRA-RED ANALYSIS

A relatively small molecule, such as this 'solvent' would appear to be, might easily be identified by infra-red spectral analysis of the grease, supposing it to be markedly different from the molecular configuration of the wax molecules. However, an examination of grease samples taken directly from the surface of freshly moulted animals did not indicate the existence of anything other than long-chain saturated paraffins, with CH_3 and OH groups; there was a possibility of an aromatic ring compound. Grease could not be obtained in sufficient quantity to make this approach of further practical value.

If the cuticular grease does contain some volatile materials however, these would be evaporating continuously—though probably in minute quantity—from the surface of living animals. Accordingly, a culture of several hundred cockroaches was established in a glass tank, kept as clean as possible, and fed on sugar, dry bread and water, thus attempting to reduce to a minimum the volatile materials from the food. The tank was sealed, except for one air inlet through which air was drawn via a filter; at the other end, air was removed through a polythene tube and passed through a trap immersed in solid carbon dioxide. A further filter was used to guard against any oil vapour diffusing back from the pump moving the air stream. A small volume of a watery fluid was collected each day for about 3 weeks, and kept in a sealed container; it smelled very strongly of cockroaches. The odour could be removed by adding an absorbent material such as a clean piece of porous pot or activated animal charcoal; by subsequent chloroform extraction of the absorbent, and evaporation in a micro-distillation apparatus, a small quantity of an acrid, tarry substance was obtained. This appeared to have no wax-solvent properties when a small fragment of beeswax was added; it was very slowly volatile, and it seemed most unlikely that it could be the component responsible for the dilution of the wax to a grease.

The remaining liquid appeared to consist almost entirely of water; it was repeatedly extracted with chloroform in a separating funnel, and the residues were distilled to a small volume at room temperature in a micro-distillation apparatus under reduced pressure. This material was again subjected to infra-red spectral analysis; apart from chloroform, it showed only the presence of long paraffin chains, CH_3 and OH groups.

SUGGESTED NATURE OF SOLVENT

Chibnall *et al.* (1934) have shown that two insect hard waxes consist of entirely saturated long-chain paraffins, alcohols and smaller quantities of acids and esters. Beament's comparative study (1945) of beeswax with other epicuticular waxes indicates that most of these could consist of a similar series of compounds, i.e. the even-numbered members of the aliphatic series, covering a wide range from perhaps C16 to C36.

The evidence presented above indicates the presence of solvent materials of low molecular weight, compared with the hard waxes, but chemically identical with them. Now it would be most unwise to assume that such a solvent would consist of one discrete chemical entity, or indeed of a very few components. It is, in fact, more than likely that the solvent would be made up of the same chemical species which are considered to compose the hard wax, but that the various series extend down into the range where they are volatile and liquid at temperatures in the biotic range. The pertinent properties, for even-numbered carbon-chain members of the alcohol, paraffin and acid series are given below; it seems likely that these are the only ones which insects would produce, and that plants produce the alternate members of the series (Chibnall *et al.* 1936):

Chain length	Paraffin	M.w.*	B.p. (° C.)	Alcohol	M.w.	B.p. (° C.)	Acid	M.w.	B.p. (° C.)
C 4	Butane	58	Gas	Butyl	74	117	Butyric	88	163
C 6	Hexane	86	69	Hexyl	102	157	Caproic	116	205
C 8	Octane	114	125	Octyl	130	195	Caprylic	114	Solid
C 10	Decane	142	174	Decyl	158	231	Capric	172	Solid
C 12	Dodecane	170	214	Dodecyl	186	Solid	—	—	—
C 14	Tetradecane	198	Melts at 5° C.	—	—	—	—	—	—

* M.w. = molecular weight.

In addition to these properties, one must of course, bear in mind the water solubility of the compounds, for unless their lipoid solubility is most pronounced, they will not in all probability be useful in a grease directly in contact with a water-saturated cuticle. It is immediately seen that if the acids are part of the solvent material, they would have to be of very short-chain length to be liquid at biotic temperatures. They are then very water-soluble, and it is shown below that the lower members of the acid series are not good wax solvents.

It is further interesting that long-chain acids do not make up more than a few per cent of beeswax, according to Chibnall's analysis, and that COOH groups were not recognizable from infra-red analysis. Perhaps most important is the very strong and characteristic smell of the lower aliphatic acids—this is certainly not detectable when the grease is heated, and weighs most strongly against the presence of acids in the solvent.

Of the alcohols and paraffins, hexanol is appreciably soluble in water, but octyl alcohol has a much greater affinity for oily materials; decyl alcohol is the highest member of the series which would be sufficiently liquid at room temperatures to 'liquefy' a wax. On the other hand, higher chain lengths of the paraffins are still liquid, and even tetradecane is just liquid in the biotic range of temperatures. Hexane, on the other hand, is extremely volatile, and octane would seem the lowest member of the series to be of possible interest.

If it is true that in the cockroach, the manufacture of paraffins and alcohols extends in each series down to chain lengths sufficiently short to be liquids, then the production of a 'solvent' is not at all difficult to understand. Whether the grease contains similar amounts of each of a series, or whether there are larger amounts of particular members is not clear. It does, however, seem that there is a disproportionate amount of short-chain materials in the grease—on the evidence of molecular weights, and it is possible that they are derived from intermediates in the process of building up the long-chain waxes.

Further information can be obtained from the rate of evaporation of a chloroform extract of grease. If this is plotted against time, and compared with recordings of the loss of chloroform from a saturated solution of beeswax of similar surface area and under identical conditions, one can allow for the evaporation of chloroform from the grease, and thus obtain an indication of the pattern of the rate of evaporation of the solvent. Grease samples varied quite considerably in the amounts of volatile material which they produced. It was quite obvious that evaporation of two kinds took place: a larger quantity evaporating less quickly and representing up to 40% by weight of the grease, and a more volatile one which might be 15% in a solvent-rich sample.

MODEL EXPERIMENTS

The further investigation of this problem is made difficult by the lack of micro-chemical methods for the identification of waxes and the minute amounts of fresh materials available, especially as they must always be contaminated with the substance used to extract them. A comparison of the properties of the natural grease with those of a synthetic grease compound from a readily available wax—beeswax—and paraffins and alcohols of suitable length as solvents will provide another approach. Such a comparison would be more justified if even the hard waxy part of the cockroach grease could be obtained in larger quantity; although what little evidence we have suggests that Chibnall's analyses for beeswax do apply to other epicuticular waxes, the peculiarities of the cockroach grease might make it a little dangerous to assume that this also consists only of compounds such as are found in beeswax.

COCKROACH WAX IN QUANTITY

If a cockroach comes into contact with any porous and absorbtive material, some of its mobile grease may be lost. The cultures of cockroaches in the Zoological Laboratory at Cambridge normally contain a number of inverted flowerpots of unglazed earthenware, in nests four to six high, to provide a shelter for young stages, and a rough surface for attachment of animals during moulting. Cockroaches appear to avoid a clean flowerpot, placed freshly into a tank; they are rarely to be seen on one as compared with those pots which have been in the culture tank for a few weeks. An attempt was made to extract the less volatile components of the grease from such flowerpots. A number of pots were first thoroughly washed in chloroform, to remove any fat-soluble materials in them, and left in the culture. They were withdrawn after 3 months, and placed in a large glass vessel, where they were repeatedly washed with distilled water, removing a quantity of debris and no doubt a certain amount of water-soluble materials. They were then allowed to dry and were repeatedly extracted with boiling chloroform. The material obtained on evaporation of the chloroform was a brownish very soft waxy substance. From a sample of this about half a gram of a white wax was obtained by recrystallization from boiling acetone. The material had a melting-point of 53–56° C., as compared with 56° C. for the wax left by evaporation of the natural solvent in air. The very fact that such a large quantity of wax can be readily obtained from flowerpots can only be explained by assuming that it is the non-volatile component of the copious grease on the animal. It seems most unlikely that much oil-soluble material would come from the excreta of the animals, or from the food which is placed in the tank. This material is therefore considered to be the cuticular wax in the experiments which follow, but as an additional check, a sample of beeswax, similarly prepared by recrystallization, has been included in these tests. It will be seen that there are no fundamental differences in behaviour between the two substances.

SYNTHETIC GREASE

It was first decided to investigate the properties of solutions of waxes dissolved in various combinations of octane, decane, octyl and decyl alcohols.

Solubility. When either beeswax, or the cockroach wax obtained as indicated above, are added in excess to chloroform, benzene, carbon disulphide or diethyl ether, saturated solutions are obtained, in which the liquid portion is not greatly different in viscosity from the pure solvent. Any excess wax remains undissolved, and if evaporation is allowed to take place, wax is correspondingly thrown out of solution. An accurate figure for the solubility of the wax in the solvent can be determined. But no such figure is possible when any of the four supposed solvent materials are used. Both insect waxes appear to be completely miscible with each of these solvents, either alone or with mixtures of alcohols and paraffins. There is no such thing as a 'saturated solution', and if the wax is present in excess, a gelatinous mass is formed. The process of solution is slow at room temperatures.

A long-chain alcohol or paraffin as a solvent has thus a unique property in relation to these insect waxes, which are turned into greases similar to that found on the surface of the cockroach. Furthermore, as evaporation occurs, a grease made up of beeswax and octyl alcohol, for example, merely becomes more viscous; if left at room temperatures the melting-point of the mass rises and approaches that of the original wax asymptotically. Only a material miscible in all proportions can behave in this way. It is even more striking that a sample of pure paraffin wax, m.p. 56° C. does *not* form a grease in octane/octyl alcohol mixtures, but behaves as beeswax in chloroform, etc.

So far as beeswax was concerned, there seemed to be little difference between the behaviour of the paraffin or of the alcohol, or of mixtures of the two, though probably due to its greater viscosity, the corresponding alcohol formed solutions rather less rapidly; there was no significant difference in solution rates at 50° C. The cockroach wax, on the other hand, was certainly more soluble in a mixture of alcohol and paraffin than in either alone. The rate of invasion of a solid wax block is perhaps a poor indication of the affinity of the materials concerned, but it is most important to note that in a corresponding series of experiments, the saturated acids are not efficient solvents as compared with the alcohols and paraffins, nor do they form 'greases' when the wax is present in excess.

Evaporation of synthetic greases. Octane, and to a lesser extent, decane, are very volatile materials at room temperatures; octyl alcohol is by comparison much less volatile. Their rates of evaporation from a synthetic grease may however be expected to be very much slower, since they would have great affinity for the molecules of wax. Standard samples of greases containing various solvents were spread on sheets of clean aluminium foil and allowed to evaporate under controlled conditions at 20° C. The rate of evaporation of natural grease initially obtained from chloroform washings was also recorded under these conditions, but a correction was made for the amount of chloroform evaporating, using as controls solutions of beeswax in chloroform and of beeswax in chloroform and octane. From regular weighing of the foils, the following conclusions were reached.

The rate of evaporation of octane when present in about equal proportion to wax, i.e. the same proportion as the natural solvent at its maximum, is still rapid as compared with the loss from natural grease under similar conditions. The evaporation of decane is appreciably slower, while that of octyl and decyl alcohols is well within the range of the natural materials. However, there is already evidence for believing that in the natural grease there are two components, one of which evaporates more rapidly than the other. A solution of beeswax in octyl alcohol and decane, in which the proportions are 4:1:4 shows a similar pattern of evaporation, and the loss of solvents is only about twice as rapid as the loss from fresh samples of natural material.

If the original assumption—that the solvent consists of a series of compounds of the two species, paraffin and alcohol, running continuously into the range where they are solid waxes—is correct, then the addition of higher members, such as dodecane, dodecyl alcohol, to the eight and ten carbon solvents would certainly give a pattern of evaporation almost identical with the natural materials. Unfortunately pure dodecane and dodecyl alcohol were not available.

Synthetic grease and water. The grease of the cockroach is found on a cuticular surface saturated with water; it is also known (Ramsay, 1935; Beament, 1945) that it will spread rapidly over water. Since the hard wax part of the grease cannot do this, the effect of adding solvent on the spreading of our synthetic grease on water is obviously of importance. In view of the evidence (Lees & Beament, 1948) for the dispersion of wax in water in Géné's organ of the tick, by its association with a protein, one must also discover whether these materials could act as dispersants, for the wax in Géné's organ seems likely to contain solvent materials, but no water-dispersion takes place once the linkage to the protein has been broken.

All the materials investigated are lighter than water; solutions of the waxes, either in octane or in octyl alcohol alone, merely form a floating mass. In neither case does vigorous shaking produce an emulsion. But a solution of wax in both alcoholic and paraffinic components behaves remarkably when added to water in a test-tube.

Providing the glass is thoroughly clean, so that its surface is fully wetted by water, a thin film of wax spreads up the sides of the tube, often to a height of half an inch. From the mobility and properties of this film, it would seem that it is entirely supported by a water film underneath it, against the glass. An explanation of this phenomenon is not immediately obvious. Octyl alcohol, with its polar ending and high spreading pressure on water, might tend to form an orientated film with the wax. Octane, being non-polar would not be expected to show these properties. But one must realize that the typical wax molecule in beeswax for example, is two, and even three times as long as the octyl alcohol molecule. Where the alcohol is present in comparatively small proportion it is likely that almost the whole of it will be held in the water film, and one could suggest that such a situation would not be sufficient to disrupt the whole length of the residual attraction between the longer wax molecules. This disruption is, perhaps, the part played by the paraffin component of the solvent.

SURFACE BALANÇE EXPERIMENTS

It is obvious from this evidence that a more detailed comparison of the properties of the synthetic materials, as compared with the natural grease, could be made with a surface balance. This might also enable direct measurements of molecule size to be made. The apparatus used for these experiments is believed to be of a novel design, and a detailed description of it is given in the Appendix to this paper; it does, however, record surface pressures for given areas of film spread on a water-trough exactly as in the conventional torsion wire models.

The various materials were added to the water-surface in solution in benzene. Compression/area curves for samples of natural grease, cockroach wax, various synthetic greases and of stearic acid are shown in Fig. 1. It will at once be noted that cockroach natural grease is capable of exerting a very high surface pressure, and that its compression/area curve is not smooth. At a pressure of 35 dynes/cm. a break occurs; this is usually interpreted, in a 'mixture', as showing that some element of a compound film has been 'squeezed out' of the monolayer. It is particularly important to note that at about this pressure, a film of decane/decyl alcohol collapses; it would presumably be reasonable to infer that such a solvent would have been squeezed out of a complex film at that point. On the other hand, the materials which are left, and which are presumably the longer chain components, collapse at a very high pressure, above the collapse point of stearic acid, for example, but very close to the collapse point of the beeswax and cockroach *wax* films. Solvent may be squeezed out at above 35 dynes/cm. pressure and the film collapses above 80 dynes/cm. It will at once be obvious that these pressures, on the living cockroach, would be quite sufficient to promote spreading over the whole animal, regardless of whence the grease and solvent appear, and over a wet surfaced cuticle as well as over any small droplets of water on it; it is significant that, because these droplets are rapidly prevented from evaporating, they must be covered by a *compressed* monolayer.

STABILITY OF FILMS

Films of cockroach grease, under the best experimental conditions, remain stable on the trough for at least 48 hr., and the decrease in their area is of the order of 2% per day. The same is true for synthetic grease films. If this change is due to evaporation, then rates of loss from a monolayer may well be lower than from a corresponding thick layer on a piece of glass or tinfoil, when the exposed area is taken into account. It seems possible that the evaporation of solvent may be retarded through the polar groups' attachment to the water surface.

If a powerful emulsifier, such as the polyethylene glycol, C. 09993, is injected in minute amount into the water beneath the film, its collapse is immediate and dramatic, but the sprinkling of an elutriate of activated charcoal on to the surface (large particles may not be supported) does not seem to alter the film at all. This is surprising, but again it points to the extreme affinity between the orientated grease, with its solvent, and the underlying water (cf. Holdgate, 1955).

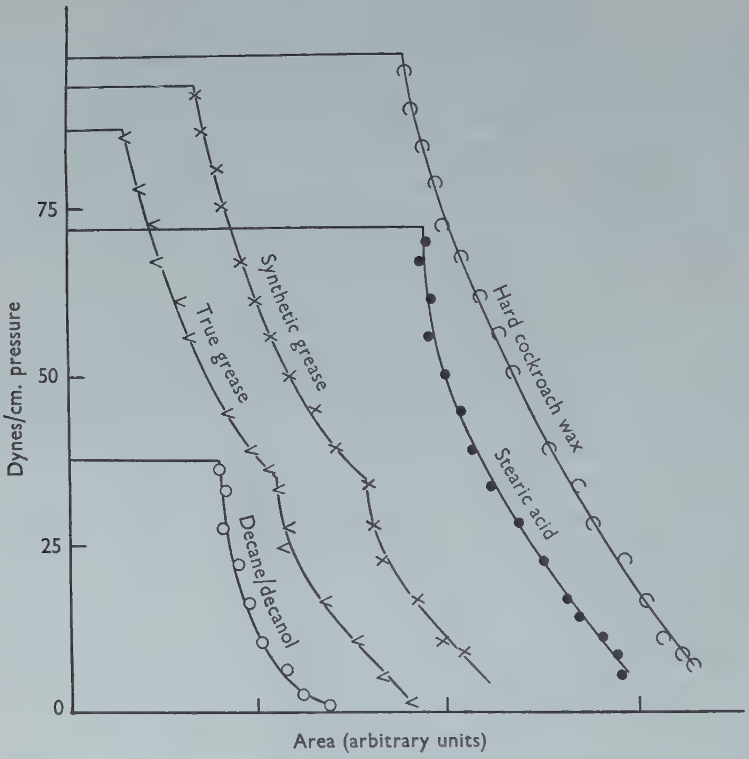

Fig. 1. Graph showing relation of surface pressure to area of monolayers of true cockroach grease, a synthetic grease of hard cockroach wax with decane and decyl alcohol, and the separate components of the synthetic grease. Stearic acid is shown as a standard. The scales of the abscissae have been chosen arbitrarily for each graph, in order to display them.

DETERMINATION OF MOLECULAR SIZE IN THE FILM

Provided one knows the amount of material added to a surface, spread as a monolayer, the area it occupies under zero compression, and its density, one can calculate the average molecular length and surface area occupied by each molecule on a water surface (Adam, 1941). With the material used in these experiments, the greater difficulty is to measure the amount of material added to the surface of the balance. This can however be approached, by carrying out a cryoscopic determination of a sample of grease in benzene, before adding a part of the same solution to the trough. The cryoscopic reading allows a calculation of the number of molecules in the sample, and thus the area and depth occupied by them when arranged in a monolayer. Results are shown in Table 3.

Table 3. *Average length and area of molecules of various waxes, determined from the area of a known quantity of wax at zero compression on a surface balance*

Material	Cross-section area at zero compression (Å.^{2})	Molecular length calculated from area (Å.)	Nearest whole number carbon chain
Hard cockroach wax (see p. 522)	24	41·5	29
Beeswax	22·1	36	25
Vaporized grease	22·4	33	23
Vapour in whole grease			
Sample E	21·9	19·8	14
F	23	18·5	13
G	22·1	20·1	14

First, it will be seen that a very reasonable figure has been obtained by this method for beeswax, bearing in mind the results of Chibnall's analysis. That the 'hard' portion of the cockroach wax is of shorter average length, is not surprising if we are right in assuming that the range of compounds in the grease may run continuously from hard wax to liquids at biotic temperatures. There would in fact be no sharp dividing line between 'wax' and 'solvent'. The mean figure for the length of natural grease molecules, as compared with a hard wax sample, supports the idea that the solvent molecules are of the order of C_{12}–C_{14} in length. It must, however, be remembered that while the alcohol portion of the solvent may always be occupying its full area in this film, the paraffin part, having no polar group, may well not be arranged in the monolayer, but lie between the upper parts of the longer molecules. This may make the values obtained above rather higher than is the true state of affairs. It has not, on the other hand, been possible to determine whether the distribution of chain lengths within the grease is uniform, or whether there are two 'peaks', one at short lengths, and the other at a much longer aliphatic chain size.

EFFECT OF SOLVENT VAPOUR ON PERMEABILITY

Wigglesworth (1945), Beament (1945), and later workers, all demonstrated that the vapour of, for example, chloroform makes insect cuticle and cuticle membranes more permeable to water, at room temperatures. Beament (1945) has further recorded that if extracted cuticular waxes are deposited on suitable membranes, from solutions in chloroform, the wax layer so obtained is not of the same order of impermeability to water as a corresponding natural cuticle, when the chloroform has evaporated away. Wigglesworth (1945) showed that once a cuticle had been heated beyond the melting-point of the wax, it is then irreversibly changed to a more permeable state, as shown by its transpiration at biotic temperatures. An artificially waxed membrane (from chloroform solution), on the other hand, becomes slightly less permeable at lower temperatures, after the wax has been melted, and this is especially so if the membrane is kept water saturated. The wax from the tick *Ornithodorus* (Lees & Beament, 1948) cannot be made to waterproof a membrane by deposition at room temperatures from chloroform, although a relatively more impermeable layer can be formed in an atmosphere saturated with chloroform at high temperatures.

In view of this evidence, it is of particular interest to discover what effect the materials believed to be natural solvents in the cockroach would have on natural waterproofing layers of the cuticle, and on less efficient layers deposited on wax-free cuticles from chloroform. The information required is best obtained by using insect wings as membranes, for Beament (1954) has shown that water transport through normal cuticle may be appreciably complicated by the phenomenon of asymmetry. Preliminary experiments showed that cockroach wings, either before or after chloroform extraction were not asymmetrically permeable in a water gradient. Some of the experiments reported here were carried out in the type of membrane holder described by Beament (1954); the apparatus has the great advantage that the membrane can be reversed, or otherwise treated without being subject to any kind of mechanical change. However, this apparatus is for liquid/vapour gradients, and the investigation was extended here by using an apparatus to measure transport in vapour/vapour gradients; it is shown in Fig. 2.

Table 4. *The permeability of natural and artificially rewaxed insect wing membranes to a water vapour/water vapour gradient, and following various treatments*

Membrane	Original permeability (mg. cm.^{2} hr.)	First treatment	Permeability in vapour	Permeability after evacuation	Second treatment	Permeability after second treatment
Natural cockroach wing	0·23	Chloroform vapour	0·91	0·64	Octane/octanol vapour	0·29
	0·19	Benzene vapour	0·98	0·95	Octane/octanol vapour	0·31
	0·28	Heat	—	0·31	Octane/octanol vapour	0·27
	0·11	Octane/octanol vapour	0·12	0·11	Chloroform vapour	0·88
Rhodnius wing dewaxed and rewaxed beeswax in chloroform	0·49	Heat	—	0·37	Octane/octanol vapour	0·09
	0·58	Octane/octanol vapour	0·07	0·07	Chloroform vapour	0·86

The membrane is confined in a duralumin cell, between rings of neoprene of circular cross-section lying in V grooves. This has the great advantage that the membrane cannot be split by increasing the clamping pressure. Neoprene, as compared with rubber, is largely unaffected by the vapour of any of the solvents used. The cell can equally well be used to record liquid/vapour permeability. The membrane-containing cell is clamped between the ground necks of two spherical bodied flasks, having taps on the bodies at right angles to the necks. The clamping framework of electron metal (for extreme lightness) also carries two adjustable case-hardened points on a balance beam. Adjustment of the height of these points relative to the beam will bring the centre of gravity of the apparatus to the point of maximum sensitivity, just below the pivots. This adjustment must be made *after* suitable quantities of liquid have been introduced into the two bulbs, by means of pipettes passed through the bore hole of the taps. After counterweighting the apparatus to a balance point, a steel rod projecting from the beam is rested on the hook of a sensitive torsion balance, so placed that its distance from the pivots is the same as that between the centres of the two spheres comprising the bodies of the flasks. Transfer of liquid through the membrane will thus behave as though moving from the centre of one sphere to the other, and will cause the same change in moment at the measuring point of the torsion balance. The apparatus is therefore null-point and direct reading.

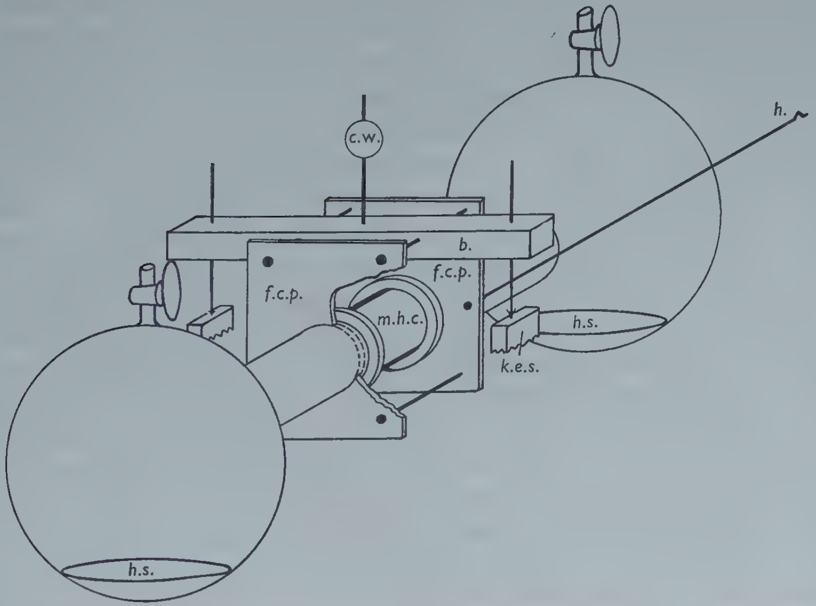

Fig. 2. Orthogonal projection of a balance to record the water permeability of membranes placed between two vapour concentrations. *b*, beam; *c.w.*, counter-weight; *f.c.p.*, flask clamping plates; *h.*, hook attached to torsion balance; *h.s.*, solution providing required humidity; *k.e.s.*, knife edge supports; *m.h.c.*, membrane-holding capsule.

In these experiments, sulphuric acid was used as the desiccating liquid on one side, and an equal weight of water gave a 'saturated' atmosphere on the other. It is important in introducing these not to allow them to splash the sides of the flasks, or to run on to the membrane and its cell. After a short period for pressure equilibration at the chosen temperature, the taps of the two sides are closed, and recordings of weight change are taken every 24 hr. The taps are opened momentarily every week to equilibrate any pressure differences which might build up between the two sides. Following the determination of the vapour/vapour permeability of a membrane, one of the various solvent materials was added in equal minute quantity to both flasks. In this way it was hoped that the weight changes recorded would not include the transport of any solvent, which would be present as 'saturated vapour' on both sides. After such recordings, either the membranes, or the humidity controlling solutions could be reversed, the membrane evacuated to remove solvent, and then recordings repeated. (See Table 4.)

So far as any topical fat solvents are concerned—chloroform, benzene, ether, etc.—their vapour makes a normal insect wing cuticle more permeable, whether this be a membrane of the cockroach or of a hard-waxed insect such as *Rhodnius*. After exposure to the treatment, even if the membranes are kept in a high vacuum for several days in the hope of removing all traces of the vapours, their permeability is still permanently increased, presumably due to a permanent disorientation of their innermost monolayer. But the vapours of octane, and of octyl alcohol do not produce any detectable change in permeability, either when experiments are conducted in their vapour, or when the membrane has been evacuated following exposure to either. There can be no doubt of the ability of these substances to penetrate into the lipoid layer of the cuticle, and even if they transform this into a 'grease' it is still apparently quite as impermeable as the original wax. The results obtained with cuticles where wax has been laid down from chloroform on a lipoid-free insect wing membrane are perhaps even more significant. These become *more* impermeable to water, and approach more closely the order of impermeability of their natural counterparts after they have been exposed to the vapour of the aliphatic solvents. That this is not due merely to the formation of a layer of grease above the solid wax deposit is again shown by the impermeability of such membranes after they have been subjected to high vacuum to remove as much aliphatic solvent as possible. It would be difficult to consider the efficiency of octane as compared with octyl alcohol in these experiments, but there is no doubt that a mixture of the two is most efficient.

Thus again, out of the range of solvents used here, it is only the vapour of a long-chain paraffin (or alcohol) that does not upset the impermeable structure of a natural cuticular wax layer. Further, such materials appear capable of producing orientation in longer wax molecules, and of reorientating a lipoid layer after the vapour of other solvents has caused misalignment. It must be remembered however (see discussion), that this evidence has been obtained with membranes, one side of which is exposed to saturated water vapour, and which therefore might contain a large amount of water.

CHEMISTRY OF GREASE

The whole of the foregoing experimental investigation into the composition of the grease of the cockroach and its properties has been conducted by physico-chemical methods, and physico-chemical models. It was therefore somewhat disturbing to find that a sample of the grease is a very strong reducing agent. In the original hypothesis, one has assumed that the composition of the lipoid materials followed the same pattern as that of beeswax, and that they were entirely saturated compounds. Since the behaviour of beeswax (which is entirely saturated) dissolved in saturated solvents, is so close to that of the grease, and as there are no signs of the phenomena usually to be associated with the 'drying oils' in the grease, it may be suspected that the reducing materials are secondary products, and not components playing any important part in the formation or composition of the grease. The hard white cockroach wax, obtained after recrystallization from the flowerpots in the culture tanks has no reducing properties; an attempt to determine an iodine number for a sample of this wax left no doubt that it was a saturated substance. A few initial experiments, using unsaturated alcohols of the order of C_8 chain length, again indicated that these were by no means as efficient as solvents as their saturated counterparts. A sample of grease obtained by washing large nymphs or adults with chloroform would reduce ammoniacal silver nitrate, giving a brownish deposit. It would also blacken rapidly in solutions of osmic acid. Following either treatment on the grease, if chloroform was added, a grey 'solution'—probably a dispersion—was formed which would pass through a fine-grade filter-paper. The dispersion could not readily be aggregated by centrifuging. A sample of grease which has been obtained freshly and changed to a hard material by heating in a vacuum—so that no oxidation could take place—still has strong reducing properties. This is of particular importance for it must surely eliminate any possibility that the solvent is also the unsaturated material. It was first thought that polyphenols and quinones might be responsible, for Pryor (1940) has shown that these are the main precursors of the tanning of the cuticle of the cockroach. Polyphenols are much more lipoid-soluble than water-soluble, and it would not be surprising if they dissolved in the grease from the underlying epicuticle substrate which is so rich in them. But no reaction could be obtained with ferric chloride, which is a most sensitive test.

It was therefore decided to try to fractionate and test for reducing materials at each stage. A large sample of grease was extracted with chloroform from flowerpots; the odoriferous material was removed by adding charcoal and filtering. Apart from the difficulty of obtaining large quantities by washing living insects, the pot extraction ensures that one does not wash reducing materials out from underlying living cuticle. From the brown residue, obtained after the chloroform had been evaporated, the wax was taken up in boiling acetone, and the brown residues were concentrated by hot filtration. This material was soluble in warm ethyl alcohol, and showed very strong reducing properties. About 100 mg. were obtained. On cooling, and allowing the alcohol to evaporate, such a solution would form a delicate pale brown skin over the surface of a dish. It was insoluble in the cold in many lipoid solvents, such as chloroform, diethyl ether, benzene and carbon disulphide, and only slightly soluble when they were boiled, though cloudy dispersions could be formed.

The properties of this material were to some extent reminiscent of shellac, and a series of tests were carried out comparing the reducing residue with a sample of pure shellac from *Laccifer lacca*. The solubilities, forms of precipitate produced when water or acetone was added to an alcohol solution of similar concentration, were so completely similar as to leave little doubt that this material was a form of shellac. A micro determination of the iodine number gave a reading of 12, which compares with 10 for a sample from *L. lacca* (Parry, 1948); the material was strongly acid which is true of shellac. Finally, treatment with warm methyl alcohol and 3 % hydrochloric acid, followed by solution in ethyl alcohol to which diethyl ether is added, produced a fine white crystalline precipitate, with a melting-point of about 140° C. Parry (1935) outlines this test for a dihydroxycarboxylic acid in shellac which has a melting-point of 149° C.

SHELLAC

Shellac is a material whose properties are far better known than is its composition, and such analyses as have appeared (Parry, 1935, 1948) indicate that it is a very complex substance. It is best regarded as a 'molecule for molecule' secretion of carbohydrate—laccose—with wax. The term laccose certainly includes a number of compounds, at least four of which have been identified, in the secretion of *L. lacca*. The amount of wax in the shellac of that animal is given as 3–5 %, but one is left in doubt as to whether this is wax, integral to the shellac secretion, or whether at least some part of it is an extraction of the waterproofing layer of *L. lacca*. The literature does not give any indication of the part of the epicuticle with which the shellac secretion is to be homologized, but it is here suggested that it is the cement layer of the epicuticle.

In the cockroach, the cement (Kramer & Wigglesworth, 1950) is a thin sheet, possibly discontinuous, and floating in the grease. It is certainly understandable that some of it would be lost to the surface of porous material over which the animal crawls; certainly no other source for the shellac in extracts of the flowerpots can be suggested. The properties of the laccose undoubtedly enable one to explain many of the problems already encountered by workers on cuticle composition (see particularly Wigglesworth, 1947, 1948). In 1945, Wigglesworth and Beament first indicated that whereas boiling solvents would readily remove the waterproofing layer of wax from insects with hard waxes, cold solvents were not efficient; this has always been attributed to the 'protective action of the overlying cement layer'. The problem became more difficult to understand through a later, unpublished discovery that a solvent, at room temperature, but boiling under reduced pressure was almost as efficient in removing the wax from a cuticle. It has also been recorded (Wigglesworth, 1948) that whereas one can see a thin sheet of material lift from the surface of the cuticle when broken down by corrosive materials, one cannot find such a substance in the solution of extracted waxes, though Beament (1945) has drawn attention to the unidentified residues in the extracted waxes of many different species, and, in particular (Lees & Beament, 1948), to the very unusual platelets of material which separate from the much lower melting-point wax on cooling a chloroform extract of cast skins of *Ornithodorus*. These cannot be made to recombine with the wax for re-waterproofing an artificial membrane and they have poor solubility in all but boiling solvents.

Now many of these problems can be solved if one assumes that the cement layer is a shellac. Presuming that only a boiling efficient wax solvent can penetrate into the wax component of shellac, it breaks up the cement by mechanical action, thus allowing the main underlying wax layer to be dissolved. The laccose is dispersed, and as we have seen in the cockroach, it is so finely divided that it will readily pass through a filter-paper; it would therefore eventually settle in the extract. The reaction of the carbohydrate portion of lac with ammoniacal silver nitrate (the argentaffin test of Lison 1936) is so similar to the reaction of polyphenols as to suggest that it might be the cuticular tanning material, in the absence of a ferric chloride test. It is no wonder, therefore, that if cement is generally composed of shellac-like substances it has not been obtained as an intact lamina by using a wax solvent. The effect of shellac on artificially waxed membranes might yield valuable additional evidence here, but in the absence of micro-chemical tests for laccose, the development of this thesis must be an extremely difficult matter. Nevertheless, it is so typical of nature that what one insect—*L. lacca*—produces in quantity, others produce in minute amount, and certainly no other source of these materials in the cockroach can be envisaged.

DISCUSSION

The oenocytes in the blood of, for example, *Rhodnius* which has a hard, cuticular wax, are cyclicly active cells (Wigglesworth, 1933). They appear to be responsible for the secretion of the lipoprotein and wax of the cuticle at moulting, but they are quiescent at other times in the moulting cycle, unless wound-healing and repair processes are going on. But in the cockroach, Kramer & Wigglesworth (1950) have described how these cells differ in their behaviour as compared with the 'typical' insect by becoming permanently re-incorporated into the epidermis at an early stage of the animal's nymphal life, and apparently remaining active continuously. Are we to conclude that in the cockroach the wax is produced only discontinuously, at the moment in ecdysis where the typical insect is secreting its wax, and that these cells then feed further solvents to the surface, to make up for evaporational loss throughout the instar? A grease can of course be made up from a small number of compounds of intermediate molecular size, which are just solid at room temperatures, or by the admixture of hard waxes (larger molecules) with liquid solvents (smaller molecules). The latter is certainly the case in the cockroach and the duration of the adult stage, and very probably, that of the nymph, is sufficient to lead to a large amount of evaporation of solvent, with a detectable change in transition point; yet preliminary work does not indicate that this takes place, and the replacement of solvent seems most likely. The continuous production of wax and solvent would equally lead to an increase in transition temperature, and the accumulation of large amounts of wax on the surface of the animal.

Before we consider the implication of wax solvents on the general problem of wax secretion, it is perhaps worth considering briefly whether the presence of a fluid grease has any particular significance to the cockroach. Fluid greases—particularly the egg waterproofing material of ticks (Lees & Beament, 1948) could be considered as biologically essential; for the egg this is obvious, and for the adult it may well assist in re-waterproofing the surface after the enormous distortion caused by engorgement. But as we have every reason for believing that the cockroach secretes its grease over its entire surface, just as other insects do, there is no reason why its wax should be more mobile initially. If the animal is subjected to temperatures above its transition point, one can attribute some importance to the fluid grease as a survival mechanism, for the grease becomes permeable and evaporation ensues, with consequent cooling (Edney, 1953). When lower temperatures prevail, the importance of the solvent in promoting reorientation has already been demonstrated. While the advantage of instantaneous repair from abrasion is obvious, such a phenomenon is not widely found, and the cockroach does not live normally in circumstances where it is liable to this sort of damage. Again, though Wigglesworth & Beament (1950) have shown that the ootheca is not waterproofed, and it now seems likely that the spreading of grease over this structure from the female, augmented by her repeated rubbing of the limbs over it, provides its only waterproofing, surely this is an adventitious process, and not one which requires a fluid grease to be present at all stages in the life of both sexes.

The functional importance of solvent materials in promoting the organization of a tightly packed waterproofing monolayer cannot be over-stressed, for it is this very organization which we hold to be responsible for waterproofing in the hard-waxed cuticles. This takes place on a water-saturated membrane, and must be contrasted with the observation (Beament, 1945) that the grease takes about 6 hr. to spread over a *dry* insect cuticle before a waterproof layer is produced. It is probably true to say that the presence of a water-saturated surface is a most essential part of this process, and that the apparent anomaly of depositing wax in the presence of water must now be considered a necessity—in the cockroach. Holdgate (1955), discussing the stability of the contact angle of grease films on the cockroach, demonstrates that the water in the substrate is an essential part of this process. We can only make the briefest comment on the likelihood of a solvent mechanism being present in other insects. The rapidity with which their waxes become solid (Wigglesworth, 1947, 1948; Lees & Beament, 1948; Beament, 1951) must surely indicate that any solvent materials present during the process of secretion must be very much more volatile than those in cockroach grease. But their presence would do more than merely ensure a properly orientated monolayer; it has always been a source of speculation that the waxes separate into a layer distal from the polyphenolic materials during secretion. It seems very likely that the spreading pressure of such polar materials would appreciably assist in the separation process, for from such a mixture they would always come to occupy the outer, surface position. A solvent might also provide the most acceptable explanation for the way in which waxes can pass through the hardened cuticle during repair processes.

SUMMARY

An investigation has been made into the properties of the grease layer which waterproofs the cuticle of the cockroach *Periplaneta americana.* This grease changes slowly into a hard wax if stored in air. The melting-point rises during this process by 20° C.

It is shown that the grease consists of a hard wax (m.p. 56° C.) dissolved in a 'solvent' which is liquid at room temperatures; there may be equal amounts of wax and of solvent. It is suggested that the series of paraffins and alcohols which probably compose the wax extend into the short-chain lengths, C_8–C_{12} to provide the solvent.

A micro-freezing-point method for determining the molecular weight of wax samples is described (based on Ramsay's method for aqueous solutions); the molecular weight of the solvent lies between 120 and 170, and of the hard wax—300–350. A method is also outlined for collecting large quantities of cockroach wax.

Of a large range of lipophilic liquids, which dissolve waxes, only octane, decane, octyl and decyl alcohols are miscible in all proportions with insect waxes, and form synthetic greases with beeswax and with the hard wax of the cockroach. The surface-active properties of the natural and synthetic greases have been compared on a surface balance of novel design which is described in the Appendix. The two materials are remarkably similar; the surface balance has been used to confirm the size of the solvent molecules.

An apparatus is described which enables the continuous measurement of water transport through an insect membrane in a water vapour/vapour gradient, and in the presence of other vapours. As opposed to the effect of the most organic wax solvents, the vapour of an octane-octyl alcohol mixture does not alter the permeability of a natural insect cuticle to water; exposure of an artificially waxed membrane to this vapour and water increases its impermeability. Evidence is provided for believing that octane-octyl alcohol mixtures will improve the waterproofness of most 'imperfectly' waxed membranes.

The natural grease of the cockroach is a strong reducing agent, and evidence is produced for believing that the lipoid part is entirely saturated, but that it contains a small portion of substances resembling shellac. It is suggested that this represents the 'cement' layer of the cockroach cuticle.

The importance of spreading agents with wax solubility in the formation of insect waterproof layers is discussed; it is suggested that the presence of a water-saturated substrate is an essential part of this process.

I am particularly grateful to Prof. V. B. Wigglesworth, C.B.E., F.R.S., who provided the material with which this investigation was started, for his constant interest in the work. Mr G. D. Glynne-Jones kindly provided me with a quantity of pure beeswax. I have had valuable discussions with many members of the Zoological Department, Cambridge, and wish especially to acknowledge the advice of Dr R. H. J. Brown, Mr M. W. Holdgate and Drs M. G. M. Pryor and J. A. Ramsay.

APPENDIX

A surface balance for investigating the properties of monolayers

Following the original work of Pockels (1891), Langmuir (1917), Adam & Jessup (1926) and Lyons & Rideal (1929) have been mainly responsible for the development of 'surface balances'. In all their models, the principle adopted has been that of a very delicate float against which the monolayer film exerts its pressure. Measurement is carried out by a null-point method, the counterbalancing of the pressure on the float by the rotation of the torsion wire on which the float is suspended. Workers in this field are agreed that the principle is not without its disadvantages: the torsion fibre is temperature-sensitive; the calibration of the float involves loading it in a different way from its normal measuring constraint; the robustness of the float system is inversely proportional to the sensitivity of the fibre; the eventual measurement is subject to the interpretation of a geared tensioning device. Further, this is a measuring system which is very sensitive to unbalanced pressure with respect to the centre of suspension of the float, and any such unbalance is going to alter the overall tension in the measuring system. It seemed possible that many of these objections could be overcome by using a beam and knife-edge principle for measurement. The apparatus described below is shown in Fig. 3, and was used to determine the surface pressure information which is given on page 526.

A frame, constructed throughout in $1 \times 1 \times \frac{1}{8}$ in. brass angle, rigidly brazed at all joints, carries the trough of water on the surface of which measurements are to be made. Three levelling screws enable the trough edge to be made horizontal. The frame also carries the main pivot mechanism: an agate plate, carried in an aligning cage, and operated by a lever system spring-loaded against a fine pitch screw. The pivot can thus be adjusted to a given height; the beam is then freed to swing by dropping away the side supports, but it is essential (see below) that the actual vertical travel of the beam, in this process, be as small as possible. It is also an advantage for the beam to have a small swing. The beam centring and dropping devices must be placed well away from the knife edge (as opposed to typical balance design) in order that it shall be completely free of interference for small angles of swing. All parts are constructed of brass throughout, to minimize corrosion in the damp atmosphere over the trough. The beam carries the float against which the film exerts pressure. Now a knife edge can safely carry a load in excess of 200 g., so that the float could be robustly constructed of a casing of 0·001 in. brass shim, filled and coated with pure 65° C. m.p. paraffin wax, bonded on to the smooth metal with a layer of dried dilute Bostick. It is supported by an entirely triangulated framework of thin-walled $\frac{1}{8}$ in. diameter stainless steel tubing, attached so as to allow vertical and horizontal alignment of the float with the line of the knife edge. The float-supporting structure meets the float entirely above the water line of the trough; thus the float presents vertical waxed surfaces at all points of contact with the water surface. The best barriers between the ends of the float and the side of the trough were found to be the thinnest tissue paper impregnated with warm pure petroleum jelly.

The beam is notched at accurate intervals, and forces are applied to the film by adding riders of known weight to the beam. A simple calculation of moments about the knife edge gives the force per unit length of float for a known load on the beam. The compression barrier is moved by a double screw and chain drive and the beam carries a plain mirror, to obtain zero positions by lamp and scale.

Experimental procedure. After cleaning and sweeping the trough, the beam counterweights are adjusted to obtain a zero under free swinging conditions. The balance is clamped, barriers placed in position, the surface checked for contamination and the sample, in its volatile solvent, applied with an 'Agla' micrometer syringe. A suitable weight is added to the beam, the balance arm freed, and the compression barrier screwed along until the light spot returns to zero; the area of film is then measured. The rider is moved to produce a greater moment and the operation repeated, thus giving the normal force/area curve. Restriction of the vertical movement of the beam is essential, since it is well known that dipping a vertical surface through a compressed monolayer will produce deposition of films on it, and thus remove an area of film from the surface at each operation.

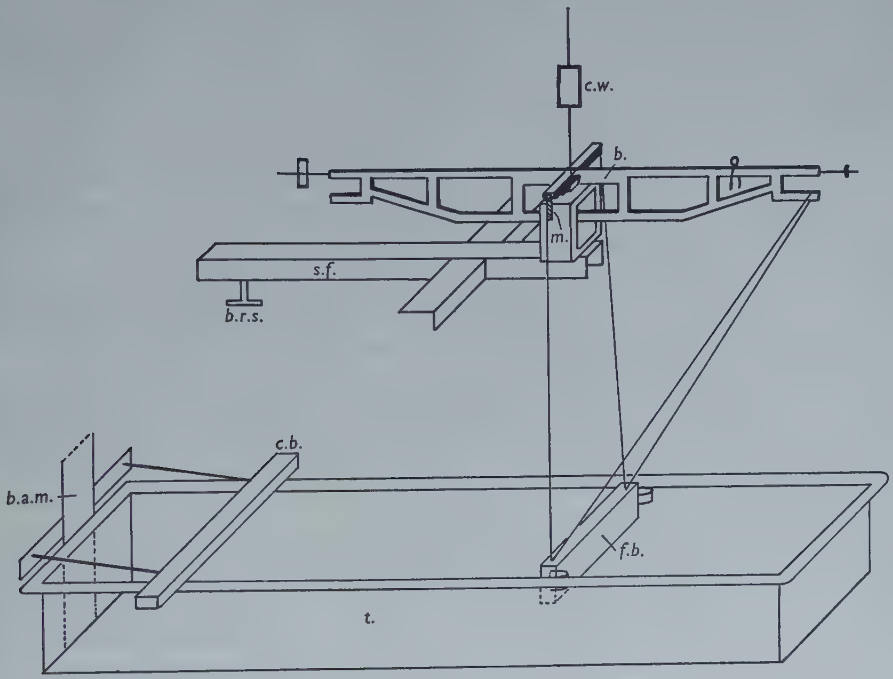

Fig. 3. Orthogonal projection of a surface balance working on a beam principle. *b.*, beam; *b.a.m.*, barrier advancing mechanism; *b.r.s.*, beam release screw; *c.b.*, compressing barrier; *c.w.*, counterweight; *f.b.*, floating barrier; *m.*, mirror for lamp and scale; *s.f.*, supporting framework; *t.*, trough.

Sensitivity. A properly constructed beam balance on a knife and plate, suitably adjusted for centre of gravity, will respond to a displacing force of one dyne. This effect can easily be displayed by the lamp and scale. A dyne at the beam is equivalent, in this model, to 0·45 dyne over an 18 cm. float, i.e. 0·025 dyne/cm. This is, of course, well beyond the required accuracy of measurements of the kind indicated below; it is perhaps more important to note that 4 g. at the beam counterbalances a film of 100 dynes/cm., which can be measured accurately within 1:1000.

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MEMBRANE POTENTIALS IN THE MUSCLES OF THE SOUTH AMERICAN FROG, *LEPTODACTYLUS OCELATUS*

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The experiments described here were made in Brazil during 1953. Their primary purpose was to explore the properties of the muscle fibres of the locally available frog, *Leptodactylus ocelatus*, and to find out whether, in spite of the very different climate to which the animals are accustomed, their isolated muscles provide as convenient experimental preparations, and give similar results to those obtained, for instance, with the sartorius of European *Rana temporaria*. This was found to be the case, and in addition to repeating a number of established effects on *Leptodactylus ocelatus*, some new results obtained with changes in the ionic composition of the solution will be reported. Intracellular recording of resting and action potentials was used and the conditions were chosen so that the experiments could be compared with those made by other authors. In addition, an investigation of the effect of some of the variables upon the rate of extrusion of sodium from the muscle fibres was measured by radioactive tracer methods; these results will be reported later.

METHODS

The potentials were measured and recorded by using glass microelectrodes (tip diameter 1–2μ) filled with 3 M-KCl (Ling & Gerard, 1949) and connected to a high impedance cathode follower unit and a direct coupled amplifier. The preamplifier and input stages were similar to those described by Bishop & Harris (1950). An oscilloscope was used when it was desired to record action potentials (A.P.); the resting potentials (R.P.) were read off a meter connected across the pre-amplifier output. A source in series with the input permitted calibration to be carried out with the electrode *in situ*. Readings quoted are the mean of at least six penetrations, the S.D. is given or indicated on the figure.

The sartorius muscles of *L. ocelatus* were dissected from small specimens, muscles weighing over 200 mg. being unsuitable because they soon deteriorate, probably on account of inadequate oxygenation. The sartorius was attached to a piece of thread at each end, and to one end was tied a short piece of rubber thread, this enabling the muscle to be held by means of clips with slight stretch across a Petri dish containing the saline solution.

The basic solution employed was of the following composition: Na 120, K 2, Ca 1, Mg 1, Cl 96, HCO_3 30, HPO_4 1, mM./l. When the K concentration increased this was effected by adding KCl to a constant total volume because this reduces or prevents swelling (Boyle & Conway, 1941). Other solutions are specified in the text. The solution was stirred by bubbling with oxygen +5% CO_2.

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RESULTS

Connective tissue coating. The muscles when freshly dissected were put in the solution and observed microscopically in order to see the penetration of the microelectrode. It was found that on the inner surface of the sartorius there was a layer of transparent gelatinous material, a thread of which could sometimes be drawn up by the microelectrode out of the solution. The thickness of the layer seemed to increase with time, and it is likely that it swells in the saline solution, thus contributing an increasing fraction to the extracellular volume of the muscle. The presence of this coat probably accounts for the fact that after the composition of the solution was changed more than 10 min. was required for the potential to become stable even though the microelectrodes were put into the outer layer of fibres to which the solution has most ready access. The mean diameter of the fibres in the living muscle was about 80μ; this was based on a count of the number of fibres seen side by side across the calibration of a 2 mm. graduated eyepiece. The fibres appeared to run the whole length of the muscle (2–3 cm.).

A series of measurements was made to compare the potential when the muscle was in the standard solution with the potential in a solution containing more (or less) than the standard concentration of K. Fig. 1 shows the results obtained for three muscles whose R.P. in 2 m-equiv./l. solution was 100 mV. within 2 mV. Other muscles showed R.P.'s in this solution varying from specimen to specimen, though muscles from a particular frog were very similar. The range of values from various frogs in the 2 mM. solution was from 85 to 103 mV. Readings were taken at 23–27° C. In an experiment in which the temperature was reduced to 6° C. the reduction of potential was less than the standard deviation. In one specimen the potential rose as the potassium concentration was reduced below 1 mM., but in three others it remained nearly the same as it had been in the 1 mM. solution. The form of the membrane potential/K concentration curve is similar to the one reported by Jenerick (1953) for the muscles of *Rana pipiens*. It is noteworthy that in a saline mixture having 147 mM. of K there is still a potential of about 16 mV.

A series of measurements was made using mixtures of 0·1 M-Na and K phosphates (each one having a composition intermediate between mono- and di-basic salts). The muscles twitched in these mixtures if less than 10 mM. K was present. Potentials obtained were always lower than those found in the same muscle in a saline mixture of K concentration equal to that of the phosphate mixture ($>99\%$ significant by t test). In pure K phosphate mixture ($K=150$ mM.) the potential was 4–6 mV., and in the dibasic salt ($K=200$ mm.) it was difficult to tell when a penetration had been made; the membrane potential was certainly less than 4 mV.

In pure KCl the potential was lower than in a saline mixture containing the same K concentration, in 147 mM.KCl the value was 8 ± 1 mV.; in 300 mM.KCl it was between 0 and 4 mV. In pure $KHCO_3$ (170 mM.) a value of 8 ± 2 mV. was obtained.

Anion changes. A comparison was made of the R.P. in three different chloride-bicarbonate mixtures (Table 1). The K concentration was 2 mM. in each. The figures suggest that the R.P. may increase with the proportion of HCO_3 and the consequent increase of pH. More alkaline mixtures could not be used because the muscle twitched spontaneously.

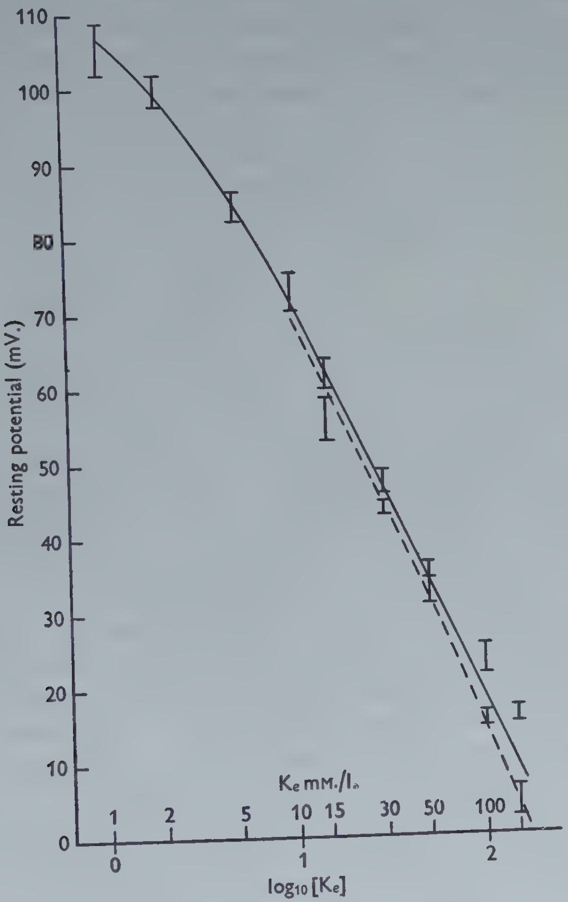

Fig. 1. The resting potential of fibres of *Leptodactylus ocetalus* sartorius muscles measured (full line) in saline with various amounts of KCl added and (dotted line) in mixtures of Na and K phosphate. Measurements at 26–28°C. The vertical lines show + and − the standard deviation. The curves are drawn according to figures calculated from the constant field equation (see text).

The potentials in the phosphate mixture, as already stated, were lower than in the saline mixture of the same K concentration.

Attempts were made to replace Cl by other anions. Bromide could be used giving a R.P. = 88·5 ± 2 mV., the control in the usual chloride mixture was 90 ± 5 mV. The bromide solution did not cause continuous spontaneous activity. It was noticed that on penetrating a fibre in the bromide solution there would be a local contracture which disappeared in a few minutes. This could not be attributed to gross damage since the same electrode would later give high and steady R.P.'s. Stimulation in the bromide solution caused long-lasting contractions.

Both nitrate and sulphate if used to replace all the Cl (but leaving the HCO_3) led to spontaneous twitching. Potential readings were therefore taken in mixtures in which half the Cl was replaced by its equivalent of either nitrate or sulphate. The results of two muscles (*a*) and (*b*) were, in normal saline, (*a*) = 87 ± 3 mV., (*b*) 96 ± 1·7 mV; in half nitrate, (*a*) was 83 ± 5·5 mV. (*b*) was 82 ± 1 mV., and in half sulphate, (*a*) was 87 ± 4·5 mV. Thus sulphate did not appear to reduce the R.P. while nitrate did so. This latter effect was reversible.

Table 1

	HCO_3	Cl	pH	Resting potential (mV.)
(1)	14	114	6·75	83 ± 3·5
(2)	30	96	7·1	86 ± 2·4
(3)	48	78	7·8	88·5 ± 2·0

The differences between (1) and (2) and between (2) and (3) are not significant, but the difference between (1) and (3) is significant to the 2 % level.

Other solutions. When all the Na was replaced by equivalent tetraethylammonium there was no immediate reduction of the resting potential, but this was almost certainly due to the slow loss of Na from the surroundings of the fibres. Action potentials observed during the equilibration period (i.e. before all the Na can have been removed) lasted 10 times as long as the normals. If the muscle was kept in the solution for more than 15–20 min. the R.P. began slowly to fall. It was reduced by about 10 mV. in the next 20 min. These observations lead one to the conclusion that if time is given for most of the muscle Na to be carried away by the Na-free solution the R.P. is reduced. Probably its eventual rate of fall will be determined by the rate of K leakage from the muscle which was not determined in this solution. Replacement of all the Na by guanidine also caused the R.P. to fall; the rate of fall was greater than that caused by the tetraethylammonium.

Tubocurarine in concentration 6×10^{-6} stopped nerve stimulation being effective after a delay of 10 min. It did not affect the R.P., and indirect excitability could readily be restored by washing in the ordinary saline mixture.

The effect of two metabolic poisons was investigated. Addition of dinitrophenol at a concentration of 25 mg./l. only led to an insignificant fall of R.P. in $1\frac{1}{2}$ hr. from 87 ± 2·5 to 84·5 ± 2 mV. Replacing 1/10 of the chloride by fluoride prevented indirect excitation but did not affect the R.P.

Action potential. Observations were made of the A.P. resulting from indirect stimulation. The amplitude of the 'overshoot' was 35–50 mV. Fig. 2 illustrates some responses. In Ringer's solution the greater the reverse membrane potential the faster was the rate of fall of the spike. In nitrate mixture (half Cl replaced by nitrate) the rate of fall was much reduced even when the maximum of the spike was high, while the rate of rise was not visibly affected. In bromide the action potential appeared to be sometimes lengthened as in nitrate, but in addition there was an indication of repetitive firing. These effects are being further investigated in the species *Rana temporaria*. *Added in proof*: In *R. temporaria* the slow rate of fall has sometimes been seen in ordinary Ringer, so it cannot be regarded as a specific result of nitrate treatment.)

Electrolyte composition. Samples of plasma were analysed for Na, K and Cl. Values obtained were (in μ-equiv./g. plasma): Na 122, K 4·8 (mean of 6, range 2·1–6·7), Cl 90·5 (mean of 3). The variability of K content may be due to variations in the time since the last meal or to the presence of various parasites.

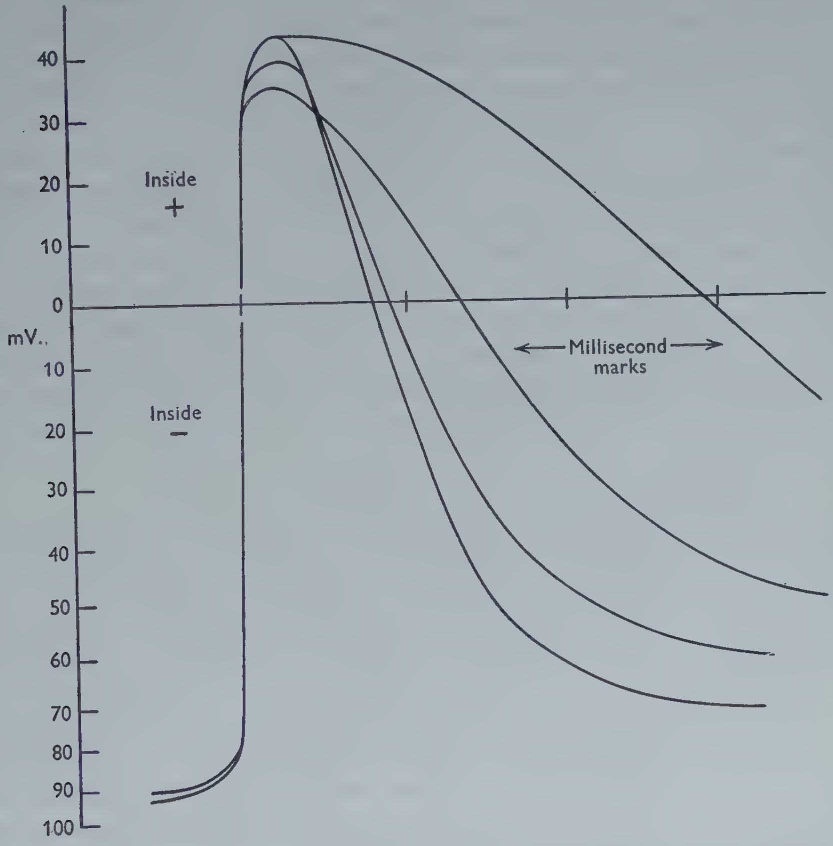

Fig. 2. Tracings of action potentials from *Leptodactylus* fibres. The three faster responses are taken in chloride-bicarbonate solution; note that the rate of decay varies according to the amplitude of the positive overshoot. The slow response is typical of those obtained in a solution with half Cl replaced by NO_3.

Na and K contents, dry weight, and Cl and SO_4 spaces were estimated on the muscles. The dry weight was 19·2 ± 0·9% of the wet weight. The K content of freshly dissected, unsoaked muscles was rather variable, ranging from 99 to 85 μ-equiv./g., and the Na content ranged from 37 to 32 μ-equiv./g. The Cl in freshly dissected muscle corresponded to a Cl space of 32·3%, which exceeds the Na space (26·2–30·3% based on the above plasma and muscle Na values). The excess Cl may be explained either by presence of intracellular Cl or by combination of Cl with basic extracellular material. The latter is favoured by the fact that the SO_4 space of soaked muscles was also rather high. If we take for calculation a dry weight of 19·2% and an extracellular space of 30% the internal K concentrations would be between 167 and 195μ-equiv./g. cell water in the fresh muscle.

Measurements of the space accessible to Cl and SO_4 were made in muscles exposed to either the normal saline mixture, or to one having half the Cl replaced by SO_4 for periods of 1 hr. The Cl space was 33·2%, i.e. a little greater than the fresh muscle value which was obtained on specimens taken from the same frog. The sulphate space, determined by chemical estimation of the SO_4 liberated from a previously soaked muscle, ranged from 28 to 37% (mean of 11 = 32·8%). It is unlikely that SO_4 enters the fibres because muscles do not swell in isotonic potassium sulphate solution.

The K-content of the soaked muscles was a little lower than that of the fresh muscles, it ranged from 72 to 92μ-equiv./g. (mean of 14 = 83·9μ-equiv./g.). The corresponding internal K concentration comes to 176μ-equiv./g.

Experiments, made in parallel with the potential measurements, on the kinetics of the loss of radio Na from previously treated muscles also permitted estimation of the space occupied by the fast-moving component of the Na according to the method used by Harris & Burn (1949) as well as of the internal, or more slowly moving Na. The extracellular space by this method was 27–35% and the internal Na concentration came to about 7μ-equiv./g. cell water.

DISCUSSION

The behaviour of the resting potentials as a function of the external K concentration is very similar to that found for other frogs. The resting potentials found in certain solutions permit the theoretical calculation of the relative permeabilities to Na, K and Cl ions using the Goldman (1943) constant field solution of the diffusion equation. One has for the potential difference between inside and outside

$$E=\frac{kT}{e}\ln\frac{[K_i]+r_1[Na_i]+r_2[Cl_e]}{[K_e]+r_1[Na_e]+r_2[Cl_i]},$$

where r_1 is the ratio of the Na to the K permeability and r_2 is the ratio of the Cl to the K permeability. Following the observation of Conway (1947), that muscles did not swell in $KHCO_3$ solution, it will be assumed that bicarbonate does not enter as such. Phosphate has been shown not to penetrate muscle by Eggleton (1933) and later workers, e.g. Harris (1953).

In a pure KCl solution and before there had been time for appreciable penetration of K along with Cl the application of the equation is simplified because Na_e is zero and r_1Na_i proves to be negligible. To give 8 mV. in 147 mM. KCl r_2 comes to 0·17, the value is little affected whether we take $[Cl_i]$ as 1 or 10 mM. Turning now to the saline mixtures a good agreement with experiment can be obtained if one takes r_1 as 0·015; the most doubtful figure in making the calculation is the Cl_i, which has been assumed constant at 1 mM. The magnitude of this latter figure somewhat affects r_1, and it has to be supposed that [Cl_i] does not increase during the time necessary for the K ions of the external solution to equilibrate with the solution in the immediate vicinity of the fibres. Jenerick (1953) made this same assumption. The value for r_1 fits the results obtained in the phosphate mixtures, in which the Cl ions do not present any problem. It is worth remarking that the differences of R.P. found between various muscles in a given solution must arise from differences of r_1 rather than of Na_i and K_i since the value of the fraction is much more sensitive to changes in r_1 than to changes in Na_i and K_i.

The values of r_1, r_2 found for *Leptodactylus* do not differ greatly from those found by Jenerick (1953) for *Rana pipiens* (he found $r_1 = 0{\cdot}027$ and $r_2 = 0{\cdot}23$).

The effect of the anions on r_1, r_2 is of interest. Can the permeability ratios be changed by changes of solution composition? Nitrate does appear to reduce the resting potential, which might be explicable by a changed r_1, the reduction of the [Cl_e] term being insufficient to account for the observed change. Br and SO_4 ions do not appear to alter r_1 in the resting state.

The prolongation of the A.P. seen in nitrate solution could be explicable by a change of the permeability ratios applying in the *active* state, thus if the K permeability were reduced it would take longer for the polarization to be restored by an outward K current. Present preliminary results of permeability studies do not indicate that the resting state permeabilities are affected. Here it may be noted that the recent observation of Carey & Conway (1954) that there is less entry of K from a sulphate mixture than from a chloride mixture into *R. temporaria* muscles does not show clearly that sulphate depresses K permeability. The observations shown in their fig. 5*A* and *B* are confined to a period of 100 min. and only indicate that the initial rapid entry of K amounts to exchange of about twice as much alkali ion when in the chloride as it does when in the sulphate. Tangents at 80 min. do not show greatly different slopes.

An influence of Br and NO_3 ions in lengthening the duration of the active state of muscle contraction has been described by Sandow & Mauriello (1953) and by Hill & Macpherson (1954). Nitrate has also been investigated by Ritchie (1954). While our observation of the slowing effect of nitrate on the recovery from the action potential may be related to the effect on the mechanical response, it does not suffice to account for the very much longer duration of the mechanical response in the nitrate medium.

SUMMARY

1. The resting potential of the fibres of the South American frog *Leptodactylus ocelatus* has been measured using microelectrodes.

2. In 2 mM. potassium Ringer solution values of 85–103 mV. were obtained. The value for a given muscle depends upon the external K concentration and the relation between resting potential and the concentrations of K, Na and Cl in the medium can be fitted to the constant field solution of the diffusion equation.

3. Use of phosphate solutions in place of chloride solutions led to lower resting potentials at a given K concentration, which is understandable since the

contribution of Cl ions to the resting potential is not present in the phosphate solution.

4. The action potential was observed. Its rate of fall was less when the positive overshoot was low than when the overshoot was high.

5. Analyses of muscles and plasma were made.

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THE SOURCES OF ENERGY FOR FLIGHT IN MOSQUITOES

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Reserve deposits of fat and glycogen are known to serve as sources of energy for flight in various insects. Among the Diptera only glycogen has been shown to serve this purpose; the respiratory quotient of *Drosophila* in flight is close to 1·0 (Chadwick, 1947), and during flight glycogen disappears from the fat body but the deposits of fat are untouched (Wigglesworth, 1949). *Tabanus* has also been shown to use fat body glycogen during flight while apparently making no use of the fat deposits (Hocking, 1953).

Mosquitoes have three possible sources of flight energy in meals of nectar and of blood and in the reserves carried over from the larval stage. The abundant literature on the flower-visiting habits of male and female mosquitoes has been reviewed by Hocking (1953), who considered that in northern Canada mosquitoes obtain energy for flight almost entirely from nectar. An indication of the extent to which reserves and ingested blood can provide energy for mosquito flight is given in the work described here.

MATERIAL AND METHODS

A strain of *Culex pipiens* form *berbericus* Roubaud, obtained in Algiers, was found to provide suitable material for study. The mosquitoes were largely anautogenous (i.e. requiring a blood meal for ovary development) and selected for anautogeny, stenogamous (i.e. able to copulate in a small space), man-biting and non-diapausing, and could easily be cultured in the laboratory. The larvae were fed on a thick bacterial infusion which developed over ground dog-biscuit soaked in water.

The mosquitoes were flown on a 'flight mill' similar to that designed by Hocking (1953) to measure the distances flown by small insects under experimental conditions. A tapering arm of steel shim, 0·004 in. thick, revolved about a vertical steel needle which passed through a hole in the arm. The arm was supported on the needle by a glass cone which was secured over the hole, the only friction as the arm revolved coming from the contact of the point of the needle with the glass cone. The tip of one end of the arm passed through a circle of 1 m. circumference and the other end was shortened and balanced with a counterpoise. A mosquito was attached to the tip of the arm by wire 0·008 in. thick. A spot of 42° C. wax was melted on to the end of the wire and this was then melted against the top of the thorax of the mosquito using a cautery of suitable temperature. When the free end of the wire had been fastened to the tip of the arm, small adjustments to the wire placed the mosquito in the normal attitude of flight. The same arm was used in all experiments, each revolution being recorded when the arm blocked light falling on to a photocell connected to an electric counter. The mosquitoes were flown at temperatures of 20–22° C. and the saturation deficiency of the air was not allowed to exceed 15 mm. Hg.

The suspended mosquito would start to fly when its legs lost contact with a surface and if flight stopped before the mosquito was exhausted it could be started again by touching the legs. The distance flown by a mosquito before it was exhausted was used as a measure of the energy available for flight. A mosquito was considered to be exhausted when for some minutes it had been unable to fly more than a few revolutions after brief rests. Most individuals flew readily on the mill and the distances and speeds recorded showed that attachment to the arm did not greatly impede flight. No attempt was made to correct the results for artificial drag so the results recorded, which were used for comparative purposes, indicate the distances flown on the mill.

In experiments in which glucose solution was fed, females aged 40–72 hr. were flown to exhaustion, kept overnight and exhausted again the following day. They were then fed 10% glucose solution from a graduated, wax-lined pipette. The mosquitoes could generally resume continuous flight within a minute of starting to feed.

THE RESERVES AS A SOURCE OF FLIGHT ENERGY

The fat body cells of fully grown larvae of *Culex pipiens* contain discrete droplets of fat and protein and deposits of glycogen (Boissezon, 1930, 1932). These reserves are carried over to the adult stage where they disappear from the fat body as they are used. There is no development of an 'adult fat body' of different structure as in the higher Diptera, but feeding on sugar leads to a heavy accumulation of fat and glycogen in the fat body cells. Staining the flight muscles shows the presence of small amounts of glycogen; the apparently heavy staining of fat is confined to the sarcosomes.

Mosquitoes which had been flown to exhaustion were fixed in Baker's formaldehyde calcium, divided sagitally in two and stained in Sudan IV, or were fixed in Carnoy's fluid, divided and stained with Best's carmine. No differences could be found in the extent of fat body fat in exhausted and unflown specimens, but the glycogen had almost disappeared from the fat body of exhausted specimens. This suggested that, as in *Drosophila*, glycogen was the principal source of energy for flight.

The fat body is fairly extensively developed in newly emerged adults of *Culex pipiens*, form *berbericus*, particularly in the abdomen where fat body lobes lie between the other organs. To find whether the fat body reserves could make an appreciable contribution to flight, females aged 48–72 hr., which had been confined before flight in 3 × 1 in. glass tubes with moist filter-paper, were flown to exhaustion on the flight mill on each of five successive days without receiving any food. They were returned to the glass tubes between flights. The results, which are summarized in Table 1, series C, and given in full in Appendix I, C, showed that considerable distances could be flown on the flight mill on the reserves alone, and doubtless much greater distances would be covered in free flight. The mean total distance flown was

5339 metres. Nearly half of the total distance was covered on the first day, the distance falling precipitously on the second day and then continuing to decline gradually.

Ten females of *C. pipiens* form *berbericus* were fed a total of 0·5035 mg. glucose after being flown to exhaustion and the sum of the distances flown on this glucose was 21,823 m. Details of the flights are given in Appendix II. By comparing the distance flown by unfed females with that of females fed glucose solution, it is found that the flight reserves of an unfed female aged 48–72 hr. must be, very approximately, equivalent to 0·12 mg. glucose, a result which agrees remarkably closely with the calculated reserves of certain species of *Aëdes* (Hocking, 1953).

Table 1. *Distances flown by females of* Culex pipiens *form* berbericus *on the flight mill*

Series	Conditions before flight	No. flown	Mean distance flown on five days (m.)					Total
			1st	2nd	3rd	4th	5th	
A	Unfed; free in cage; aged 72–96 hr. at first flight	10	1282	455	314	242	129	2422
B	Fed blood; free in cage; aged 72–96 hr. at first flight	10	1564	1809	1055	410	225	5063
C	Unfed; confined in tube; aged 48–72 hr. at first flight	10	2728	781	736	548	546	5339

Hibernating mosquitoes are known to develop particularly extensive fat bodies containing, in *C. pipiens*, droplets of fat and glycogen but no protein (Roubaud, 1933). To find the extent of their powers of flight, females of *C. pipiens pipiens* L were removed from hibernation in a cellar during December, raised to room temperature and flown to exhaustion. Most individuals were flown within a few hours of being taken from hibernation, nearly all within 12 hr. The mean distance flown on the mill was 4300 m., the maximum 8674 m. and the minimum 2239 m. The mosquitoes flew fairly readily and strongly compared with non-diapausing mosquitoes and speeds up to 1 m./sec. were recorded. This was also the maximum speed found in *C. pipiens* form *berbericus*. The females of *C. pipiens* form *berbericus* required 0·099 mg. glucose to cover the mean distance flown by hibernating females of *C. pipiens pipiens* i.e., 4300 m. It was not known whether the mosquitoes had fed on plant juices before entering hibernation. Females of the hibernating generation of *C. pipiens pipiens* have been seen feeding on plant juices before entering hibernation (Lacour, 1937), but Roubaud (1933) considered that the hibernation fat body could be developed without adult feeding.

INGESTED BLOOD AS A SOURCE OF FLIGHT ENERGY

In the absence of experimental evidence opinions have varied on the possibility of ingested blood being used as an energy source by mosquitoes. It has been claimed that there is no evidence that insects can utilize proteins to provide energy for flight (Hocking, 1953), yet it has also been stated that in the field all the indications are that females of *Anopheles* feed on blood alone (Muirhead-Thomson, 1951). However, females of *A. aquasalis* Curry have since been reported feeding from flowers (Senior White, 1952) and it is well known that Culicine mosquitoes are frequent flower visitors.

To find the effect of blood meals on the duration of flight, the distances flown by unfed females of *Culex pipiens* form *berbericus* were compared with those of females fed once, to repletion, on human blood. As mosquitoes kept under only slightly different conditions before flight showed considerable differences in flying ability, the two groups were kept, as far as possible, under identical conditions. The mosquitoes were kept at first in cages of 8 in. side, those fed blood being fed on the day before their first flight. They were first flown at the age of 72–96 hr. as few mosquitoes will take a blood meal before they are 48–72 hr. old. Each mosquito was flown to exhaustion on 5 successive days and between flights the mosquitoes were each kept in a 3 × 1 in. glass tube with moist filter-paper. The results are summarized in Table 1, series A and B, and given in full in Appendix I A, B.

The mosquitoes which had taken a blood meal flew more than twice as far as the unfed females. The mean distance flown by the end of the 5 days was 2422 m. by the unfed mosquitoes and 5063 m. by the blood-fed. There is a slight difference in the mean distances flown by the two groups on the first day but it is not statistically significant. This distance was maintained, even increased, by the blood-fed females on the second day while the unfed females could fly little more than a quarter of the distance they had covered the previous day. The mean distance flown by the unfed mosquitoes continued to fall gradually over the last three flights. The blood-fed mosquitoes flew a considerable distance on the third day, although less than on the first 2 days. This distance fell precipitously on the fourth day as it had done in the second flight of the unfed mosquitoes.

The differences between the mean flight distances of blood-fed and unfed mosquitoes was 2641 m., a distance which would be covered by exhausted mosquitoes after feeding on approximately 0·061 mg. of glucose. A meal of 3 mg. of blood would contain only 0·003 mg. of blood sugars so it is clear that digestion products of the blood, and not blood sugars alone, were providing energy for flight. In *C. pipiens* form *berbericus* development of the ovaries is normally dependent on blood feeding, most females being anautogenous, and fully grown eggs are found in the ovaries five days after the females have fed. The ovaries of females which had been flown to exhaustion on the 5 days following blood feeding were found to contain the usual number of fully grown eggs, showing that both energy for flight and material for the growth of the eggs can be obtained from ingested blood.

Histological examination of the animals showed that after a single flight to exhaustion the glycogen deposits in the flight muscles and fat body were almost depleted. Twenty four hours after exhaustion there were slight deposits of glycogen in the flight muscles of unfed specimens but practically none in the fat body. In specimens fed blood before the flight to exhaustion, slight deposits of glycogen appeared in the flight muscles after a twenty-four hour rest and considerable quantities of glycogen were to be found in the fat body.

DISCUSSION

From the nature of the reserves remaining in exhausted mosquitoes it has been inferred that glycogen, not fat, is the immediate source of flight energy in these insects, while the many accounts of mosquitoes of both sexes feeding on nectar have shown this to be an important source of flight energy. The Lepidoptera, however, which also feed on nectar have been shown to use fat and not glycogen as the source of energy for flight (Zebe, 1954). *Drosophila* is known to use glycogen in flight and its fat reserves have been considered to provide energy for activity apart from flight; it was suggested that the fat could not be mobilized rapidly enough to provide energy for flight (Wigglesworth, 1949). In mosquitoes the fat reserves are more extensive than the glycogen reserves and it has been shown that during diapause the amount of fat gradually decreases, the other solids remaining constant (Buxton, 1935). This lends support to the suggestion that fat provides energy for activity apart from flight in Diptera but there is no direct evidence to support the suggestion that fats cannot be mobilized rapidly enough to provide energy for flight and it is now known that insects which consume fat during flight show similar rates of uptake of oxygen to the Diptera (Krogh & Weis-Fogh, 1951; Zebe, 1954).

The reserves of unfed females of *C. pipiens* form *berbericus* were able to provide energy for flights of considerable length, and the digestion products of blood meals were also found to provide flight energy. Mosquitoes which were first flown a day after feeding on blood flew considerable distances for 3 days. In most cases the blood was fully digested before the third flight, a time for digestion similar to that recorded in other mosquitoes (Fisk & Shambaugh, 1952; West & Eligh, 1952; Weitz & Buxton, 1953), so it appeared that reserves were available for flight 1 day after the blood meal had been digested and thereafter the flight distance fell off.

SUMMARY

Glycogen was found to disappear from the fat body during flight in the mosquito *Culex pipiens* form *berbericus* Roubaud, while no change was observed in the fat deposits. The fat body reserves were able to sustain a flight of considerable length and ingested blood could also be used as a source of flight energy.

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APPENDIX I

Distances flown by females of Culex pipiens *form* berbericus. *The mosquitoes were flown to exhaustion on five successive days. Distances in metres*

No.	1st	2nd	3rd	4th	5th	Total
A. Unfed, aged 72–96 hr. at 1st flight; free in cage before 1st flight						
65	928	52	55	107	45	1187
66	1583	655	437	302	176	3153
67	652	856	293	161	187	2149
68	1362	467	257	309	10	2405
69	1611	378	355	262	189	2795
70	1447	545	630	285	383	3290
71	740	373	302	34	24	1473
73	1763	257	254	342	42	2658
75	930	651	288	317	191	2377
82	1806	321	267	302	41	2737
Mean	1282	455	314	242	129	2422
B. Fed human blood to repletion on day before 1st flight aged 72–96 hr. at 1st flight; free in cage before 1st flight						
46	1604	20	1702	423	270	4019
47	1205	2663	1644	711	610	6833
48	2008	1860	1157	505	318	5848
51	789	1208	711	425	192	3325
56	1161	2060	132	428	26	3807
57	1270	1970	1325	454	311	5330
77	1915	1522	835	317	159	4748
80	1864	2863	664	255	126	5772
81	2276	2279	487	134	135	5311
84	1551	1645	1892	451	105	5644
Mean	1564	1809	1055	410	225	5063
C. Unfed; aged 48–72 hr. at 1st flight; confined in tube before 1st flight						
22	2289	954	892	686	534	5355
23	2459	649	819	550	469	4946
24	3350	753	762	680	439	5984
26	3205	1385	1678	401	1016	7685
60	4122	759	657	581	950	7069
61	2160	1239	306	222	205	4132
62	2607	658	625	622	478	4990
63	2376	614	605	728	384	4707
64	2685	605	553	741	593	5177
86	2023	190	460	271	397	3341
Mean	2728	781	736	548	546	5339

APPENDIX II

Distances flown by females of Culex pipiens, *form* berbericus *fed* 10% *glucose solution after being flown to exhaustion. Distances in metres*

No.	Mg. glucose fed	Distance flown
90	0·0608	3069
91	0·045	2623
92	0·0486	2282
93	0·0502	2634
94	0·0475	1808
95	0·0486	1476
96	0·0529	2427
97	0·0524	1706
98	0·054	2256
99	0·0435	1542
Total	0·5035	21823

APPENDIX III

Distances flown by females of Culex pipiens pipiens *taken from hibernation. Distances in metres*

No.	Distance flown	No.	Distance flown
2	4525	12	3133
3	3695	13	3553
4	3690	14	3367
5	5224	15	8674
6	4913	16	5399
7	2239	17	4270
8	2693	18	5802
9	2948	19	3825
10	7677	20	3634
11	4396	21	2360

THE NERVES AND MUSCLES OF MEDUSAE

II. *GERYONIA PROBOSCIDALIS* ESCHSCHOLTZ

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INTRODUCTION

Geryonia proboscidalis Eschscholtz, formerly known as *Carmarina hastata* Haeckel, is common in the Mediterranean for much of the year. It lives well in an aquarium and remains active for several hours under experimental conditions. Because of the large size and the vigorous responses to stimulation it is a convenient animal for experimental work, and as in most medusae the responses are consistent and well defined.

Since the classical work of Romanes (1885) upon some smaller species of Hydromedusae and the work of Nagel (1894) on *Geryonia*, the behaviour and the co-ordination of movements of the Hydromedusae have been largely neglected, and attention has been turned to the larger and more accessible Scyphomedusae. This paper deals with the movements brought about by different muscle systems and the transmission of the excitation that gives rise to these movements. As in most species of Hydromedusae there are several distinct responses and there is a corresponding specialization of the nervous system into distinct but co-ordinated parts which transmit excitation to the various groups of muscles. It is assumed that co-ordination of movement must have its basis in the anatomical arrangement of the nervous system, and an attempt is made to identify the observable nerve elements with the physiological pathways as determined by experiment, but the correspondence that we can find between the activities and the arrangement of the nerves is by no means complete. In no case has it been shown by experiment that a particular fibre is actually a nerve. This uncertainty makes it necessary to use cautious terms such as 'pathways of excitation' and 'system which conducts in all directions' instead of 'axons' or 'through conducting nerve net with synaptic connexions', although further work may bring the Hydromedusae in this respect more into line with the better known Scyphomedusae.

METHODS

In the following account, 'mechanical stimulation' implies a gentle touch with a seeker or bristle. 'Electrical stimulation' was applied with a pair of fine platinum wires excited by an induction coil, adjusted to produce a response only at 'break'. Electrical stimulation always involved some mechanical stimulation.

OBSERVATIONS

The contraction wave of the circular muscle

The circular muscle of the bell is responsible for the rhythmical beat. The tangentially orientated muscle fibres lie under the epithelium of the concave side of the bell and form a muscle sheet from the base of the manubrium almost to the velum. The fibres are cross-striated, and except near the rim the muscle sheet is divided into six regions by narrow areas along the lines of the radial canals. A single electrical stimulus anywhere within the area covered by the circular muscle, and a few millimetres beyond, gave rise to a single total contraction apparently identical with a normal beat. By cutting strips from the bell it was confirmed that excitation is conducted in all directions. The direction of the cut relative to the direction of the muscle fibres is not important, and the excitation passes readily across the muscle-free strips in the region of each radial canal. When a strip of tissue is made progressively narrower the contraction wave becomes blocked at a definite point, in a strip several millimetres wide. The minimum width at which the bridge will still conduct varies greatly from experiment to experiment. Romanes (1876) considered similar observations on *Aurellia* to be evidence for a plexus of nerves.

Working with Scyphomedusae, Bullock (1943) showed that it is possible to stimulate a strip of tissue at a moment when the muscle fibres are in the refractory period following a contraction, but when the conducting elements have come out of their own refractory period. In this way the refractory periods of both conduction and contraction elements can be measured separately. This experiment can be repeated for *Geryonia*. A strip 10 cm. long was stimulated at one end. A contraction wave travelled along the strip with a velocity approximately 50 cm./sec., so that excitation in the conducting system took 0·2 sec. to reach the far end of the strip. There was a short period when the conducting system had come out of its refractory state but the muscle was still refractory. A second stimulus was applied during this interval at the other end of the strip; there was a single contraction under the second electrode but a double one under the first. The shortest interval between two stimuli was 0·5 sec., for the second stimulus to be effective. This gives a refractory period of about 0·3 sec. This result can be interpreted in terms of excitation in the nerve and contraction in the muscle, but the experiment actually shows only that the conducting and contracting systems are distinct. The above result is too great because an insufficiently long strip of tissue was used and this introduced an effect from the refractory period of the muscle.

In another series of experiments the velocity of the contraction wave was measured in strips of the bell, with and without the marginal region. The summary of the results, Fig. 1, shows that there was no significant decrease of the velocity of transmission of the contraction wave after the operation. This does not exclude the possibility that in the ring nerve there may be a transmission slower than that of the contraction wave across the bell; however, it is safe to conclude that transmission in the margin of the bell is not important for the propagation of the contraction wave during co-ordination of the normal beat.

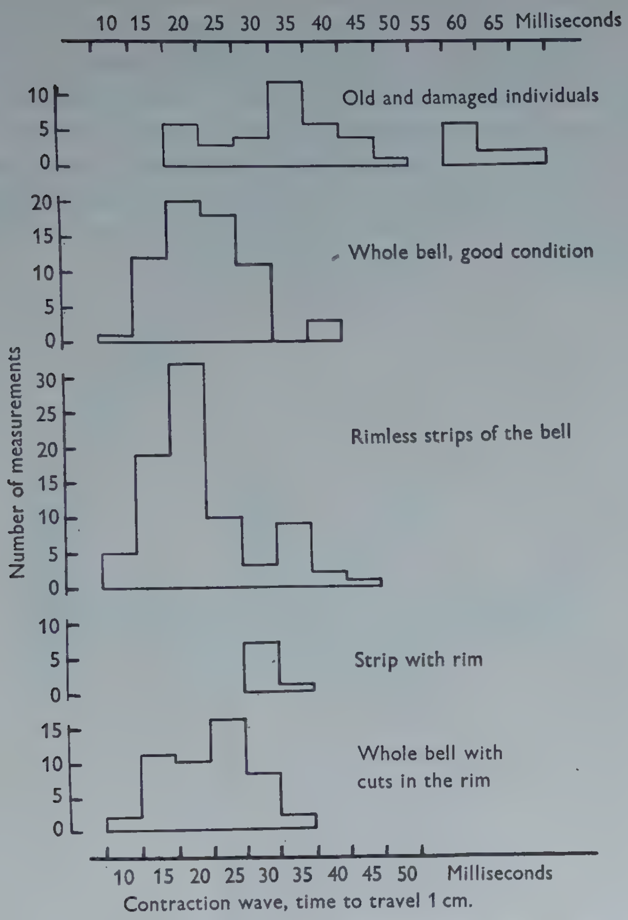


Fig. 1. Measurements of the velocity of the contraction wave. Each vertical unit represents five measurements. The velocity is not greater when the rim is intact.

The radial conducting system

Geryonia has a stout manubrium about 5 cm. long which normally hangs straight down in the centre of the bell. One of the most noticeable features of the animal is the activity of this organ, which responds to the lightest touch on the edge of the bell by a rapid and precise movement towards the side touched, and brings the terminal mouth in contact with the source of irritation. The effector muscles are arranged round the solid core of the manubrium with fibres parallel to the main axis and continue over the bell as strands along each side of the radial canals.

Preliminary experiments with entire animals showed that the mouth is quickly applied to the position of a stimulus on the underside of the bell. The response persists as long as the stimulus continues; the localization is accurate only to about 5 mm. When two places are stimulated at the same time the manubrium points somewhere between them. A tangential cut 1 cm. long through the subumbrellar epithelium blocks the inward transmission of excitation from a point peripheral to the cut. This demonstrates that the excitation cannot spread far, if at all, in a circular direction, but must reach the muscles along radial pathways. However, radial pathways alone do not provide a full explanation of the response for mechanical stimulation of the regions indicated in Fig. 2 by small letters was followed by contraction in the regions indicated by the corresponding capital letters. Stimulation near the centre of the bell produced a bending of the middle of the manubrium, but on the other hand stimulation at the margin of the bell was followed by a pointing reaction brought about by a contraction at its base.

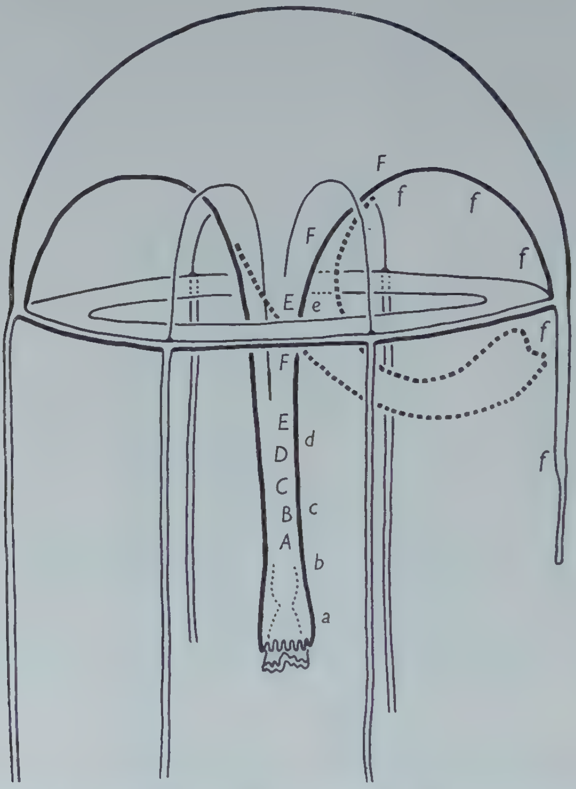


Fig. 2. A stimulation in the regions indicated by the small letters produces a contraction in the region indicated by the corresponding capital letters.

On the stem of the manubrium, experiments showed that the arrangement of pathways is not so precisely radial as on the bell. A transverse cut 5 mm. long in the epithelium of the manubrium at right angles to the muscle fibres prevented transmission along the manubrium towards the mouth. However, with strong or repeated stimulation the excitation soon spread round the sides of such a cut. It seems that here the pathways are not entirely radial, but there is a possible alternative explanation that the slow spread round such a cut is due to the mechanical stimulation by the active muscles. As the core of the manubrium is solid, muscles on opposite sides are antagonistic, so that a little spread of the excitation strengthens the response with no impairment of the accuracy of the pointing as a whole.

These results demonstrate the properties of the subumbrellar epithelium, for the co-ordinated pointing response of the manubrium is not affected by section of the six radial nerve strands, which appear to be local concentrations of physiological pathways that are generally spread over the epithelium.

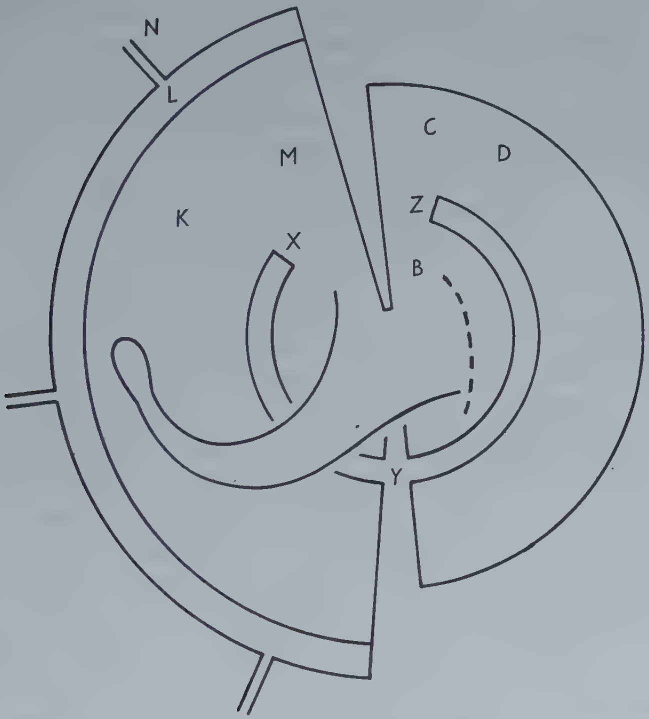


Fig. 3. Plan of the bell cut almost into two halves with two opposite radial cuts, and with two circular cuts from *Y* to *X* and *Y* to *Z*. Left side with margin and tentacles, right side without. Further explanation in text.

A preparation to demonstrate many of the characteristics of the pathways which conduct excitation to radial and circular muscles can be made from a single specimen (Fig. 3). Two radial cuts (vertical in the figure) were made reaching from the edge of the bell to the base of the manubrium, and the rim, including velum, ring nerve, and tentacles, was removed from the right half. From the lower of the radial cuts a circular cut extended into each half from *Y* to *X* and from *Y* to *Z*, almost severing it from the manubrium, but leaving a bridge on each side about 2 cm. wide.

In such a preparation electrical stimulation of one side of the bell produced a contraction wave which did not spread across the base of the manubrium to the opposite side. To the left of the dotted line at *B* electrical stimulation on the base of the manubrium failed to produce a contraction wave. A response of the manubrium followed stimulation central to the circular cuts XY and YZ and also at points M and C in a radial line with the bridges at X and Z, but not at K or D. On the other hand, if stimulation at D was electrical the resulting contraction wave spread in all directions, and although it crossed the bridge at Z had no effect on the manubrium.

The sharp distinction between the two conducting systems that carry excitation to the radial and to the circular muscles is shown by an interesting phenomenon that was accidentally noticed. A wave of contraction made to circulate continuously round the circular muscle of an undamaged specimen of *Geryonia* would continue to circulate for long periods while the bell slowly rotated in the opposite direction, vibrating all the time. Nevertheless, the manubrium would still swing out towards a touch at the edge of the bell, despite the fact that the diffuse motor pathway was continually active. This experiment has been repeated with *Liriope tetraphylla* (Geryonidae) and would no doubt be successful in other forms which have a directional response of the manubrium.

The tentacles

When a specimen of *Geryonia* is quietly floating upright the six marginal tentacles hang down from the rim. Gentle mechanical stimulation of a tentacle sometimes produced a longitudinal contraction so localized that a swelling appeared for a short time at the place stimulated. Strong stimulation was followed by contraction of the whole tentacle. Both local and propagated responses could be evoked in isolated tentacles and transmission of the excitation could occur in both directions from the point of origin. In the undamaged animal transmission was occasionally in one direction only, towards the base of the tentacle. These responses could be clearly seen by the increase in diameter of the tentacle as the longitudinal muscle contracted. Nagel (1894) distinguished between a symmetrical contraction and one which resulted in a corkscrew shape, the former due to irritants, the latter due to food juices. Judging from what I have seen the two responses are not as separate as he maintained.

The vigorous contraction of a tentacle in response to stimulation of any kind was usually followed by a similar contraction of the other tentacles almost simultaneously round the bell. This transmission between tentacles was prevented completely by making appropriate cuts through the ring nerves. When stimulation produced a contraction that reached as far as the base of the tentacle there was usually also a response from the manubrium, which would swing out towards the stimulated tentacle. The importance of this reaction in feeding is clear.

Centrifugal transmission to the margin

Gentle mechanical stimulation of the epithelium of the lower surface of the bell or the tentacles produced an acceleration of the rhythmical beat, in addition to a pointing response. In some way excitation was able to spread towards the rim and influence in a reflex manner the spontaneously active centres responsible for the rhythm. Radial pathways are indicated since the effect could not be produced by stimulation at the inner side of a suitably placed cut a few millimetres long at right angles to a radius. Shortly before the appearance of the first ensuing beat there was a co-ordinated contraction of all the tentacles. This centrifugal transmission was much slower than that for the pointing reaction in the opposite direction. There was usually a latency of 5–10 sec. between the stimulation and the response of the tentacles which was followed immediately by the beat. Since such observations could only be made with the margin entire, there was some chance of occurrence of spontaneous beats, but a consistent value of the latency was sufficient guarantee against this, and moreover spontaneous beats do not affect the tentacles. By making short incisions in the epithelium at the base of the manubrium it can be shown that in this region the pathways of centrifugal conduction, like those of centripetal conduction, are not strictly radial. Although there is a great difference between velocity of transmission in the two directions of the radial system, there is no evidence of two anatomically separate radial pathways, one going towards the manubrium the other towards the margin.

Compensatory movement and the responses of the velum

An active specimen of *Geryonia* often reaches the surface of the tank in which it swims. When this happens, as Veress (1911) and Bauer (1927) found, an asymmetry appears in the movement of the bell. The water currents at each beat now act to turn the bell, and the creature swims downwards. The same sequence happens when the water is agitated or the animal is strongly irritated.

Continuous observations of these asymmetrical beats were made by holding a specimen on a horizontal wire thrust through the main axis. Under these circumstances all specimens tended to swim downwards, and it was observed that the lower edge of the bell remained curled inwards more than the upper at the termination of each beat. As a result of this failure to relax fully, the amplitude of the subsequent contractions at the lower edge was not so great as at the upper. A considerable number of observations have convinced me that the difference is largely, and probably entirely, due to a maintained contraction of the velum. No consistent asymmetry of the contraction of the circular muscle could be observed. The bell was rotated and the observations repeated with a different sector. After each beat the velum on the lower side remained contracted and taut, whereas on the upper side it relaxed completely between each beat and waved loosely in the water current. Illumination from above or below had no effect on the behaviour. It seems that the velum at least is under the direct influence of the statocysts and may be the only effector organ of steering in this animal.

Large specimens of *Geryonia* often hang motionless at the surface of the aquarium. Close examination of the velum showed that rapid local movements appeared spontaneously from time to time; in some specimens an irregular flicker was usually noticeable. Sometimes these contractions appeared to be propagated for a few centimetres.

According to Nagel the velum is not sensitive to mechanical stimulation, but I have found a response that was sometimes quite localized and sometimes conducted with decrement for a little way round the annulus. There was no response of the bell or the manubrium. A strong contraction of the whole velum was found only at each beat of the bell. An isolated strip of the velum responded to electrical stimulation with a localized contraction only, irrespective of the intensity or frequency of the stimulation, but the contraction may be maintained for some time, which does not happen in the case of the circular muscle.

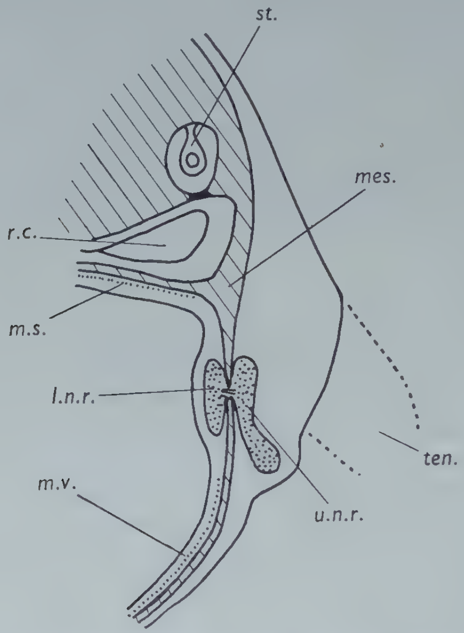


Fig. 4. The marginal region of *Geryonia*, mainly after O. and R. Hertwig, who observed the connexion between the two ring nerves in many sections. *l.n.r.*, lower nerve ring; *mes.*, mesogloea; *m.s.*, muscle fibres of subumbrellar epithelium; *m.v.*, muscle fibres of velum; *r.c.*, ring canal; *st.*, position of statocyst; *ten.*, position of tentacle; *u.n.r.*, upper nerve ring.

The structure of the nervous system

The following summary is taken mainly from the Hertwig brothers' fine monograph. There are two nerve rings, the upper separated from the lower by the supporting lamella of the velum (Fig. 4).

The upper nerve ring is a strong bundle of fibres covered with a thin ciliated epithelium to which it remains attached in macerated preparations. This epithelium contains cells of two kinds: there are sensory cells, each with a process going into the nerve ring, and there are flat epithelial cells. The upper nerve ring is divided into two strands; one part contains relatively small bipolar ganglion cells, the other part consists of thin fibres which are principally the processes of the epithelium cells overlying the nerve.

Fibres from the tentacles and from the statocysts join the upper nerve ring, which seems to be mainly concerned with the sensory parts of the nervous system. The lower nerve ring lies in a clear band between the muscle fibres of the velum and the bell. This nerve contains many ganglion cells, some of which send fibres over the circular muscle of the bell (Hertwigs, pl. 5, fig. 7). Overlying the nerve is a very thin epithelium of distinct, flat cells, among which are occasional bipolar sensory cells, each with a thin peripheral process in the surface of the epithelium.

Small bundles of nerve fibres run from one nerve ring to the other through the mesogloea of the velum. The Hertwigs found these fibres in many sections, but gave no indication of a regular arrangement. They did not find any nerve fibres in the velum, but Hyde (1902) in *Gonionemus* found some which came from the lower ring nerve.

From the base of each tentacle a radial nerve strand runs across the subumbrellar surface of the bell to the base of the manubrium. These radial nerves consist of relatively few fibres; from them axons run through the neighbouring epithelium of the bell. Over the whole of the subumbrellar surface there is a network of small multipolar nerve cells, each with three to five processes, which in the Hertwig's drawings often run in a radial direction, but these authors could not discover any differentiated endings of nerve fibres on the muscle fibres.

DISCUSSION

(1) *Conduction across the subumbrellar epithelium*

The greatest emphasis must be placed on the observation that the pointing response of the manubrium to stimulation at the margin is not impaired by the continuous circulation of a wave of contraction round the bell. The pointing reaction is brought about by radial pathways, the contraction wave is propagated in all directions, and for an anatomical basis of both these activities the experimental evidence limits us to the epithelium of the subumbrellar surface, where there is found a nerve net. Simultaneous propagation in opposite directions is not possible in a nerve or a through-conducting network of nerves in the way that electrical impulses may pass in a wire, because the refractory period prevents one impulse from passing another. The observation of simultaneous transmission seems to be conclusive evidence of two functionally separate conducting systems, so long as these are behaving in the way that other nerves are known to do. It must be remembered, however, that there is no proof of nervous activity in either radial or isotropic system.

Bearing in mind the problem of two distinct physiological pathways in one nerve net, let us examine in more detail each of the reactions concerned.

By making cuts in different directions, Romanes (1877) first showed that the contraction wave in a Hydromedusa *Sarsia* sp. is transmitted in all directions across the bell. Nagel (1894) showed the existence of the same state of affairs in *Geryonia*. These observations can be explained in terms of the network of nerve cells and their processes which can be seen in the subumbrellar epithelium. In *Geryonia* the repetition of Bullock's experiment, in which the conducting pathway was stimulated while the contractile system was refractory, indicates but does not prove the activity of nerve fibres. It has been shown that the transmission of the contraction wave in the Scyphozoan *Aurellia aurita* is by way of a through-conducting net of large axons and not from fibre to fibre of the muscle (Horridge, 1954). However, in *Geryonia*, or any of the Hydromedusae, it is only by analogy with the better-known groups that a similar state of affairs is thought to exist.

Working with *Tiaropsis indicans*,* Romanes (1877) found that the manubrium would point accurately to a stimulated place on the edge of the bell. His observations of the behaviour are detailed, and show that the movements of the manubrium depend upon a conducting system which is mainly radial. In work based directly on that of Romanes, Nagel (1894) showed that a similar state of affairs exists in *Geryonia*. The marked sensitivity of the radial pathways to gentle mechanical stimulation which does not directly initiate a contraction wave suggests that here the transmission to the muscle of the manubrium depends upon nerves closely linked with primary sense cells. The radial direction of the pathway across the muscle fibres and the centripetal polarization also suggest a nervous basis, and here the histological observations agree, for many of the nerve cells in the subumbrellar epithelium have axons that run in a predominantly radial direction.

In *Gonionemus murbachi* (Hyde, 1902) there are bipolar and multipolar cells in the radial nerve strands, the superficial ones of each kind connecting with the circular muscle and with the network of nerve cells and their processes which spread over it, the deeper ones with the radial muscle, gonads and the radial canal. The reactions of *Gonionemus* are similar to those of *Geryonia*, with marked contrast between feeding and swimming activity (Yerkes, 1902), and here is an indication of two separate parts of the nervous system. Section of the six radial nerve strands in *Geryonia* did not prevent co-ordinated pointing of the manubrium, but in this region there is certainly a concentration of radial pathways to the manubrium, as shown by stimulation with a needle electrode. In *Tiaropsis indicans* Romanes found that the mouth responded more readily to stimulation in the region of the radial canals; the same was true for *Geryonia*.

A question of fundamental importance is whether experimental demonstration of transmission in a definite direction in a nerve net is sound evidence of a structural orientation that we may expect to observe histologically. In *Tiaroposis indicans* Romanes found that the pointing reaction of the manubrium depended upon radial transmission, but was careful to restrict his conclusions. 'We must not, however, conclude that these lines are radial *structurally*: the evidence only proves that they are so *functionally*.' This is strictly true, but there is now considerably more information about the action of nerves in Coelenterates and other groups of animals. It would be likely that a nerve net with functional polarization should show some structural polarization of the fibres. For precise location of physiological pathways a direct record of the electrical activity of a nerve is most satisfactory, but in practice conclusions must be based mainly on the general agreement of separate histological and physiological evidence. The through-conducting system of the mesenteries of *Calliactis* (Pantin, 1952) is an example where the histological basis was demonstrated after the physiological behaviour was known. With all this in mind, it is clear that to understand the subumbrellar nerve net in *Geryonia* a more detailed histological study is required.

* Now known as *Eutonina indicans* Romanes.

(2) *Integration of the physiological evidence*

Light mechanical stimulation of the tentacles or the underside of the bell was followed by a response of the manubrium and a co-ordinated contraction of the six tentacles. Exploration with an electrode, and the sensitivity to chemical stimuli (Nagel, 1894) indicate that there are sensory cells widely spread over the surface. The experiments on *Geryonia* have shown that receptors on the tentacles and on the subumbrellar epithelium connect to pathways which conduct excitation in each direction along the tentacles, in each direction round the ring nerve, and radially towards the base of the manubrium. This inter-related system of physiological pathways is here called the tentacle-radial system and is sharply contrasted with the separate contraction wave system which is the basis of the co-ordination of the beat. It can now be seen that most of the physiological pathways depicted in Fig. 5 fall into one of these two groups. In this diagram an effort is made to represent the inter-relations of these conducting systems where they meet in the margin of the bell.

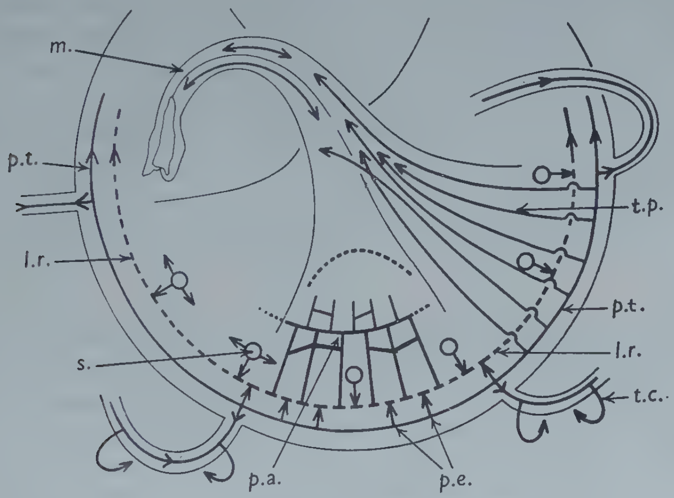


Fig. 5. Diagrammatic representation of the physiological pathways and their relations as demonstrated in *Geryonia*. This should not be regarded as a plan of the nervous system. *l.r.*, line of rhythmical centres round margin; *m*, manubrium; *p.a.*, pathways in all directions to circular muscle; *p.e.*, polarized excitation between the two systems; *p.t.*, path between tentacles; *s.*, statocyst has a local effect on velum and also accelerates the rhythm; *t.c.*, tentacle contraction; *t.p.*, radial pathways to manubrium.

Romanes (1876) showed that in all Hydromedusae studied the beat originates in the margin of the bell, probably in the ring nerves. The co-ordination of the eight anatomically-separate rhythmic centres in *Aurellia* implies that the impulse from the leading centre brings about the discharge of all the other centres and resets the rhythm in each. Pantin & Vianna Dias (1952) showed that an extra stimulus injected into the rhythm in *Aurellia* is followed by a pause of the same duration as the intervals between the other beats. In Hydromedusae the bell may be cut into small sections which continue to beat each at their own rate, indicating many rhythmical centres. Although the more refined experimental evidence is not available, it is reasonable to conclude that these centres are normally co-ordinated in the same way.

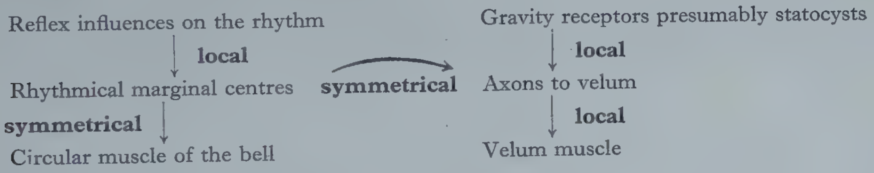
Stimulation of a tentacle or of the subumbrellar epithelium gives rise to a beat in a manner that recalls a reflex action. There is evidence that two distinct systems, radial and rhythmic, are present in the marginal nerves, and that one acts on the other in an irreversible manner. First, section of the ring nerve destroys the co-ordination between the adjoining tentacles, showing that the through-conducting tracts pass all round the margin. Secondly, the rhythmical beat has its origin in another system which is similarly distributed. Excitation in the tentacle-radial tracts in the ring nerve can influence the rhythmical mechanism and bring about a series of beats or accelerate the existing rate, but the excitation responsible for the contraction wave does not leak in the opposite direction into the tentacle-radial system. This is illustrated in Fig. 5 by short arrows from the outer circular pathway to the inner (from the tentacle-radial to the rhythmic system and not vice versa). This may be compared with the polarization of a vertebrate synapse. Observations similar to those which are the basis of this conclusion have been made by the author on a number of different species of *Hydromedusae*, and also of Scyphozoa; in some species, for example, in *Nausithöe* (Coronatae), the action is to inhibit the beat rather than accelerate it.

Turning again to the histological basis of physiological pathways in the light of the information summarized in Fig. 5, there are reasons for considering that part of the upper nerve ring corresponds with the circular pathway between tentacles. The nerve bundle which runs along each tentacle joins the upper nerve ring. The close association with overlying primary sense cells agrees with the sensory nature of other parts of the tentacle-radial system. In each of the Trachymedusae studied histologically the upper nerve ring is associated with the statocysts, although in *Geryonia* no actual connexion was described by the Hertwig brothers, but indicated by Eimer (1874). This is sufficient to remind us that the co-ordination between tentacles cannot be the sole function of the upper ring nerve.

The lower ring nerve appears to be partly motor in function, having ganglion cells which send out fibres to the velum on one side and to the circular muscle on the other; perhaps it is wholly motor in function for there is not much histological evidence of connexions with sense cells. The least complicated view of the functions of nerves of Hydromedusae is that each bundle of fibres carries only one kind of excitation, but enough has been said to show that the nervous system is not a homogeneous net of similar elements, which are sometimes concentrated into well-defined tracts. Conditions are more complicated than this for, except for the radial nerves, there is physiological evidence of differentiation in each of the nerve trunks. Local action on the velum and symmetrical activity of the bell are quite distinct but both are associated with the lower nerve ring; the through-conducting pathway in the upper nerve ring must be distinct from the local fibres of the epithelial sense cells; excitation from the tentacles can influence the rhythm and also the manubrium; the statocysts influence the velum and possibly the rhythm, and yet the nerve fibres from both tentacles and statocysts run into the upper nerve ring. The picture that is emerging is one of physiologically separate through-conducting tracts which may be superimposed on each other in a net or concentrated into mixed bundles. They meet and interact in the marginal ring nerves, though the mechanism and full extent of this interaction are still unknown.

(3) *The velum*

The complexity of the action of the nervous system is brought out by a consideration of the control of the velum, which may contract locally and is active as a whole at each beat. The contraction of the velum as a whole is not necessarily symmetrical, and it seems that this is due to a symmetrical contraction superimposed on a local one. A possible conclusion from the observations is that the through-conducting system of the contraction wave brings about a contraction of the whole velum by acting on a second localized conducting system. The action must be irreversible, for stimulation of the velum produced only a local contraction. Summarizing these conclusions we have:


Reflex influences on the rhythm
local
Rhythmical marginal centres
symmetrical
Circular muscle of the bell
symmetrical
Gravity receptors presumably statocysts
local
Axons to velum
local
Velum muscle

It is difficult to decide the function of the velum in the various species of Hydromedusae. I have also observed a local contraction of the velum independent of the beat in *Melicertum octocostatum.* The suggestion of a steering function is not new. Yerkes & Ayer (1903) expressed the view that *Gonionemus* can direct its course by contraction of the velum; Murbach (1903) was inclined to agree, and the present observations on *Geryonia* confirm these suggestions. There are two short references to the function of the velum of the freshwater medusa *Craspedacusta* (Milne, 1938). The velum contracted with the bell, restricting the outgoing water to a jet, and relaxed while the bell expanded. Secondly, animals in an inverted position would sometimes drive vertically downwards against the bottom of the tank, and were able to progress only after an asymmetrical contraction; it was the velum rather than the bell itself which was responsible for the changed direction of the expelled water.

SUMMARY

1. The responses of *Geryonia proboscidalis* to electrical and mechanical stimulation have been studied with particular attention to the transmission of excitation.

2. There are two kinds of excitation transmitted across the undersurface of the bell; first, the symmetrical beat is co-ordinated by an isotropic conducting system which acts in a through-conducting, all-or-nothing manner, and produces brief contractions of the circular muscle; secondly, the movements of the manubrium are co-ordinated by a radial conducting system, which produces a maintained contraction of the radial muscles of the manubrium.

3. The independence of these two conducting systems is shown by the simultaneous and yet distinct transmission of the two kinds of excitation in different directions.

4. The contraction wave is not transmitted through the ring nerves faster than over the rest of the bell.

5. The manubrium bends towards a stimulated tentacle, and all the tentacles contract simultaneously, co-ordinated by a through-conducting pathway in the marginal nerve. There is evidence that a polarized pathway carries excitation from this system to the rhythmical marginal centres.

6. Observations of *Geryonia* held with the axis horizontal show that the steering action is due to an asymmetrical maintained contraction of the velum.

7. The known histological details of the nervous system are compared with the physiological pathways demonstrated by experiment. The differentiation of a primitive isotropic nerve net into distinct tracts with separate functions is essential for the several rapid feeding and swimming activities.

I should like to thank Dr R. Dohrn and his staff at Naples for their kind interest and for the facilities of the Stazione Zoologica.

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THE BEHAVIOUR OF BARNACLE CYPRIDS IN RELATION TO WATER MOVEMENT OVER A SURFACE

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(*Received* 1 *January* 1955)

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INTRODUCTION

There is a close similarity of behaviour among the settling stages of different groups of sessile marine invertebrates (Crisp, 1953*a*; Knight-Jones & Crisp, 1953). Generally the process of settlement can be divided into three distinct phases, which will be called, respectively, attachment, exploration and fixation.

On attachment the larva comes into contact with a solid surface and begins crawling over it, but the process is usually reversible, since the larva retains its swimming powers. Exploration consists of one or more crawling excursions over various surfaces following temporary attachment, the intervals between these migrations being occupied by periods of renewed swimming. Fixation, however, is irreversible and determines the ultimate site of the adult; it usually involves an orientation reaction and a cementing process.

Although most marine larvae possess some ability to swim, their own movements would carry them through distances which are very small in relation to the wide expanse of sea over which suitable habitats are scattered. They therefore depend largely upon mass water movements for dispersal, and for bringing them into contact with objects on which to settle. It follows that they must be able to attach themselves to these objects under conditions of water movement similar to those which had carried them into the vicinity.

The limiting effects of water current on settlement have been studied more carefully in barnacles than in any other group of sessile animals. The exposure of settlement plates in a tideway where the current varies continually, as carried out by

McDougall (1943), may reveal differences in behaviour between species but suffers from the defect that no precise relation between current and settlement can be deduced. The most informative work on this subject is that of Walton Smith (1946) who used rotating disks and water pumped through graded glass tubes to obtain conditions of steady flow. He showed that the settlement of cyprids was inhibited by water currents of greater than a certain velocity. The critical velocity was found to vary to some extent with the species of barnacle studied. His hydrodynamical approach, however, was not beyond criticism, as explained below.

DESCRIPTION OF WATER FLOW

Since at a liquid-solid interface there is no relative movement (or slip) between the phases, the fluid velocity very near the boundary of a stationary object will approach zero, however great the water current may be some distance away.

At the moment of attachment the cyprid cannot be more than a millimetre from the surface, and since it is small and of similar density to water it will take up a velocity close to that of the fluid stream in this region. It is therefore the velocity of the water past the substratum about 0·5 mm. from the surface which will determine the possibility of attachment. We shall refer to this velocity as Δv, and the distance approached by the cypris at attachment as Δy.

Since the velocity gradient normal to the surface, $\partial v/\partial y$ is usually constant within a short distance of a stationary object, Δv will be given approximately by the relation

$$\Delta v \approx \left(\frac{\partial v}{\partial y}\right)_0 \Delta y, \tag{1}$$

$(\partial v/\partial y)_0$ being the velocity gradient at the boundary of the object. Hence it is the velocity gradient or rate of shear at the boundary of an object that is likely to be the determining factor in settlement, rather than the velocity of the water current an unspecified distance away from it. The experiments described in this paper have therefore been designed, as far as possible, to enable $(\partial v/\partial y)_0$ to be measured. The importance of choosing the appropriate hydrodynamic quantity has been overlooked by previous investigators, who have suggested that settlement is prevented if the current itself exceeds a certain speed. Although the velocity gradient varies in the same sense as the current, the relation between the two is entirely dependent upon the topography of the object in relation to the stream.

When the object is relatively small, such as a post in a wide stream, the water flow diminishes sharply in its vicinity. This region, in which the flow of fluid is influenced by the object, is called the boundary layer, for outside it the general flow remains sensibly undisturbed. Clearly, the larger the stationary object the greater the thickness of its boundary layer; in other words, the greater the disturbance of the stream flowing past it.

The work of Blasius (1908), Kármán (1921), and Lamb (1932) has led to approximate mathematical descriptions of the steady flow past a small plane lamina held parallel to the current. The thickness, h, of the boundary layer at a distance, x, from the leading edge is given very approximately by the relation

$$h^2 = \frac{2\pi^2}{4-\pi} \frac{\eta x}{\rho U}, \tag{2}$$

where U = stream velocity of fluid, η its viscosity, and ρ its density.

According to Lamb (1932), the velocity distribution given by the equation

$$v = U \sin \frac{\pi y}{2h} \tag{3}$$

leads to values of the drag consistent with the results obtained independently of this assumption by Blasius (1908). Lamb's assumed velocity distribution may therefore be taken as an approximate description of flow in the boundary layer.

Differentiating Eqn. (3), substituting h from Eqn. (2), and putting $y=0$, we obtain for the velocity gradient at the surface of the lamina

$$\left(\frac{\partial v}{\partial y}\right)_0 = \frac{\sqrt{(4-\pi)}}{2\sqrt{2}} U^{\frac{3}{2}} \sqrt{\frac{\rho}{x\eta}}. \tag{4}$$

Eqn. (4) shows that the velocity gradient over a small plate lying in the current increases towards the leading edge of the plate (as x decreases). Settlement would therefore become progressively easier at greater distances from the leading edge; moreover, the larger the plate exposed the greater the chance of settlement occurring on it. It should be noted that the velocity gradient increases with the value of the current raised to the power 1·5. If the flow rate or the size of the system is sufficiently large, so that the function $U\rho x/\eta$ exceeds the critical Reynolds number for the system, a turbulent régime becomes established. The rate of shear then increases with the velocity raised to a power approaching 2, and does not vary greatly with the length of the object. Several empirical equations have been advanced for the drag under turbulent conditions, differing from one another mainly in their application to surfaces of different roughness (see Baker, 1948). Froude's equation, based on experiments with long planks (Froude, 1872), is in common use and gives the resistance R_f as

$$R_f = fSU^n, \tag{5}$$

where f is a constant dependent on the length of the plank, S is the wetted area and n is approximately 1·85.

Assuming a thin layer of laminar flow very close to the surface we obtain for the mean velocity gradient

$$\left(\frac{\partial v}{\partial y}\right)_0 = \frac{fU^n}{\eta}. \tag{6}$$

It is clear from these considerations that the flow conditions in regions of turbulence or in the vicinity of small objects, even of those of the simplest conceivable form, are complicated and not readily amenable to exact experimental treatment. If, on the other hand, the water is caused to flow through a tube or channel with long parallel walls, the equations for non-turbulent flow are much simpler, since after the entry length is passed there is no change in velocity along the length of the tube, and each section has a similar velocity pattern. The simplest system from the experimental point of view is a long tube of circular section, to which the Poiseuille (1846) equation may be applied. The velocity gradient at the surface of the tube $(\partial v/\partial r)_0$ where r = radius is given by

$$\left(\frac{\partial v}{\partial r}\right)_0 = \frac{4q}{\pi r^3}, \tag{7}$$

where q is the quantity flowing through the tube in unit time. The velocity v at any point at a distance r' measured from the centre will be

$$v = \frac{2q}{\pi}\left(\frac{1}{r^2} - \left(\frac{r'}{r^2}\right)^2\right). \tag{8}$$

Since $\partial v/\partial r$ is not linear, Eqn. (8) may be used to obtain a more accurate value for $\Delta v/\Delta r$ than that given by Eqns. (1) and (7) above.

Eqn. (8) also shows that the velocity at the centre of the tube, v_c, is twice the mean velocity, v_m:

$$v_c = 2v_m = \frac{2q}{\pi r^2}. \tag{9}$$

METHODS OF INVESTIGATION

A rotating apparatus was first employed, consisting of a shallow circular glass trough, about 15 cm. in diameter, with a circular plate of black ebonite lying at the bottom, and just fitting it. A circular glass plate, with good clearance from the sides of the trough, was mounted on a vertical shaft and rotated by means of a pulley and motor through a variable speed gear box (Fig. 1*a*). In such an apparatus the flow is, unfortunately, not entirely tangential to the moving plate because of centrifugal effects, which cause water to veer outwards across its surface. The flow over the surface of the lower plate has a compensating inward component, increasing in magnitude (relative to the tangential flow) towards the centre of the disk (Fig. 1*b*). Computing difficulties therefore ruled out this system for quantitative experiments. It proved to be a convenient apparatus, however, for observing the behaviour of individual cyprids when exposed to continuously variable rates of shear, as existed over different parts of the disk.

By a slight modification the whole vessel and its contents were rotated with the upper glass plate removed. Cyprids swimming against the direction of rotation appeared to remain stationary when in a part of the vessel moving with an equal and opposite velocity. Then u, the velocity of the cyprid, could be measured from the speed of rotation in revolutions per second (n), and the distance of the cyprid from the centre of the dish (b)

$$u = 2\pi nb. \tag{10}$$

The majority of observations on attachment in relation to known rates of shear were made in long glass tubes of diameters ranging from 2 mm. to 1 cm. The water flow was arranged from a constant head device, and the rate of shear was obtained by measuring the volume of water issuing in unit time. Cyprids were introduced one at a time from a pipette, and their rapid movement down the tube was observed visually. This method was more successful with the larger cyprids of *Balanus balanoides* than with those of *Elminius modestus*, which being much smaller were less easy to follow.

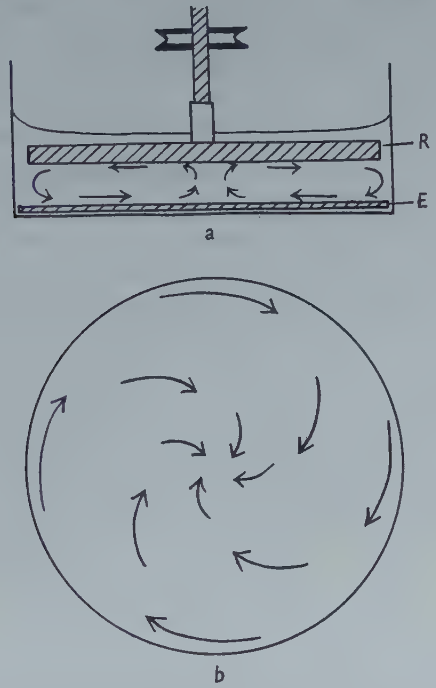

Fig. 1. (*a*) Apparatus for observing the behaviour of cyprids when exposed to current. *R*, rotating glass plate; *E*, ebonite disk. Arrows indicate the direction of water currents. (*b*) The direction of flow at the surface of the ebonite disk, seen from above, when the glass disk is rotated clockwise.

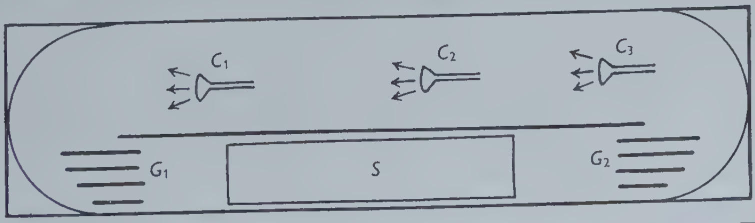

Fig. 2. Trough for experiments on settlement under conditions of steady laminar flow. The water is driven by three compressed air jets—C_1, C_2, C_3—playing on the surface, and returns through the guides, G_1, G_2, over a Bakelite strip, *S*, bearing barnacles which initiate settlement.

Experiments on settlement and orientation were made in a large trough, about a metre in length, shown in plan in Fig. 2. Water was driven up one side by means of compressed air jets and flowed passively back along the opposite side. Cyprids were introduced and their settlement was confined to strips of Bakelite bearing previously settled barnacles. These strips were placed along the side of the apparatus remote from the air jets. Since the rest of the trough bore no barnacles it was not attractive to cyprids and none attempted to settle there (Knight-Jones & Crisp, 1953; Knight-Jones, 1953). This apparatus could be left with a steady circulation of sea water for periods of up to 48 hr., and the rate of shear could be controlled by altering the depth of the water or the rate of discharge of the air jets. Particles dusted on the surface could be timed over a measured length, and the rate of shear calculated on the basis of uniform laminar flow. The effect of the curved ends of the apparatus was minimized by inserting small Bakelite guides to prevent vortices persisting.

VELOCITY OF SWIMMING OF CYPRIS LARVAE

The cyprids of *B. balanoides* and *B. crenatus* are strongly phototactic when swimming. This reaction was used to determine the velocity of swimming, by shining a beam of light into the sector of a rotating dish which was moving away from the source of light. The cyprids on certain radii then appeared stationary, and their speed was calculated as described above. The results are given in Table 1.

Table 1. *Rate of swimming of* Balanus *cyprids*

	Rotational speed of dish n (rev./sec.)	Distance from centre at which cyprids maintained position b (cm.)	Velocity $2\pi nb$ (cm./sec.)
B. balanoides	0·28	2·4	4·2
	0·28	3·5	6·0
	0·28	3·0	5·3
	0·14	4·7	4·1
	0·14	5·0	4·4
Mean	—	—	4·8
B. crenatus	0·14	4·5	3·9
	0·14	4·5	3·9
Mean	—	—	3·9
B. crenatus in surface film	0·14	6·5	5·7
	0·28	3·4	6·0
Mean	—	—	5·85

Both species can swim at a speed in the region of 4–5 cm./sec., though it is difficult to judge whether this sustained speed represents their maximum effort. They did, however, give the appearance of struggling to prevent themselves being carried away from the light source.

B. crenatus cyprids, which are slightly smaller than those of *B. balanoides*, swam a little more slowly. They showed one striking difference in behaviour in that on rising to the surface they broke the surface film very easily and appeared unable to get underneath it again. Nevertheless, they could swim in the surface film more rapidly than when submerged, owing to the reduction in the drag of the water when only part of the body was covered. This behaviour is probably quite abnormal, since *B. crenatus* cyprids usually settle at lower levels than those of *B. balanoides* and may not be so well adapted as are the latter to remain below the surface film, with which they would not normally come into contact.

ATTACHMENT OF CYPRIS LARVAE EXPOSED TO DIFFERENT VELOCITY GRADIENTS

(*a*) *Experiments using rotating plates*

Observations were first made in the rotating glass disk apparatus, using cyprids of *B. balanoides* and *B. crenatus*. When the disk was rotating at its maximum speed cyprids of *B. balanoides* were still able to attach to the lower plate and retain their hold. Rough calculation, on the basis of tangential movement alone, gave a shear of about 100 sec^{-1}, but in fact it would have been somewhat greater on account of the unknown centrifugal and centripetal components (see p. 572). Settlement occurred most readily in regions subjected to gradients of greater than 25–50 sec^{-1}. The cyprids of *B. cretanus* did not attach as readily as those of *B. balanoides*; when the upper plate was rotated they tended to collect in the centre of the dish. Few of either species attached to the glass surface, and those which did so soon swam off.

When *B. balanoides* cyprids attached to the lower ebonite surface and crawled over it, water currents produced by the upper plate did not orientate them in the manner that might have been expected, namely by swinging the body passively about the antennules on which it was pivoted. On the contrary, they appeared to be remarkably unaffected by the water movement and walked in any direction at will. At rates of shear of about 100 sec^{-1} they could with slight difficulty walk with the body at right angles to the stream of water. However, with rates of shear of this order they usually swam off after walking a short distance, two or three centimetres being covered at the most.

(*b*) *Experiments using glass tubes*

The behaviour of cyprids of *B. balanoides* and *E. modestus* attaching under different rates of shear was measured quantitatively by placing about ten larvae in a tube about 1·5 m. long, through which a measured flow of sea water was passing (see pp. 572–3). In no case did the condition become turbulent, the critical value for the Reynolds number not being exceeded. Each larva was introduced separately with a pipette, a procedure which needed a degree of patience, because the larvae frequently attached to the end of the pipette or to the outside of the tube. The progress of each cyprid was noted as it passed between a pair of marks placed 1 m. apart along the tube. If it attached, even temporarily, between these marks it was recorded as being 'successful'. If its progress between the marks was uninterrupted, the trial was recorded as a 'failure'. The percentage of 'successes' at different rates of flow are plotted in Fig. 3. It was interesting to note that at lower rates of flow the cyprids often did not swim, but were rolled passively over and over along the bottom of the tube by the current. This passive behaviour was progressively less in evidence as the rate of shear increased, and the highest percentage of attachments in *B. balanoides* occurred between 60 and 80 sec.^{-1}. When the rate of shear exceeded 50 sec.^{-1} the cyprids were stimulated to great swimming activity and often attached immediately. At these higher rates of shear the cyprids were not carried passively downstream, but swam hard against the flow, occasionally succeeding in gripping the side by an antennule and so coming to rest. Beyond 100 sec.$^{-1}$ attachment was clearly more difficult, and there resulted a steady fall in the percentage of successes, attachment never being observed at rates of shear above 400 sec.$^{-1}$. It was not found possible to obtain a current of sufficient magnitude forcibly to pull them off the tube once they had attached firmly. Nevertheless, attachment at very high rates of the order of 200–400 sec.$^{-1}$ was often momentary, as though the antennulary sucker was not able quite to complete its hold. A moderate current tended to keep the cyprids gripping firmly once they were attached, and detachment could be obtained only by reducing the flow to a sufficiently low level.

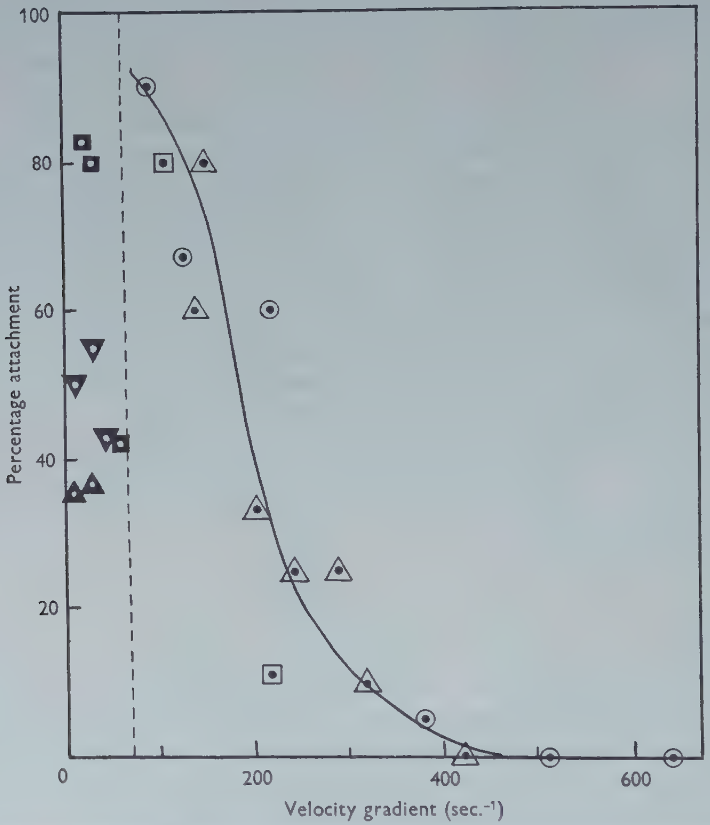

Fig. 3. Percentage of cyprids of *Balanus balanoides* which attach when passed through a 1 m. length of glass tube, plotted against the velocity gradient at the surface of the tube. ⊙, tube radius 1·13 mm.; △, tube radius 1·80 mm.; ▽, tube radius 2·65 mm.; ⊡, tube radius 3·2 mm. Open symbols indicate strong reaction to current and attempt to attach. Solid symbols indicate little or no reaction to current and desultory attachment. The dotted line indicates approximately the minimum gradient necessary to stimulate a strong attachment reaction.

Fig. 4 illustrates a similar experiment using *E. modestus* cyprids. Their behaviour differed from that of *B. balanoides* larvae in that the stimulating shear was somewhat lower, maximum attachment being achieved at less than 50 sec.$^{-1}$, and passive behaviour was less in evidence. This difference may have been due to the cyprids of

Elminius being in better condition, but it would be in keeping with ecological differences between the species, for *Elminius* settles commonly in quiet inlets and estuaries where the stimulus of water movement does not always exist (Moore, 1944), while *B. balanoides* is often found in more exposed situations. Nevertheless, *Elminius* was able to settle at high shear rates even better than *B. balanoides*. The smaller size of the cyprid may account for this difference, for other factors being equal it will be closer to the surface at the moment of attachment and therefore, at a given velocity gradient, it will be carried at a slower speed and exposed to smaller forces. *Elminius* cyprids were observed to be strongly photo-positive in a strong light after settling in the tube. If either side of the tube was shaded they walked away towards the illuminated side; similarly, by appropriate shading, they could be induced to walk with or against the direction of the flow of water. Walking against the flow of water appeared to be somewhat easier, as would be expected, since the flow tended to keep the body pressed down on the substratum. When made to walk in the same direction as the current, at high rates of shear of about 600–1000 sec.$^{-1}$, the hind end of the body was sometimes lifted, apparently by the current, and the cyprid appeared to have difficulty in bringing it down again (Fig. 5). Cyprids often swam off when the current was directed in this sense. At rates of shear exceeding 1000 sec.$^{-1}$ they appeared unable to walk with the current, and either swam off or remained in one place. They could still walk across the current, however, and did

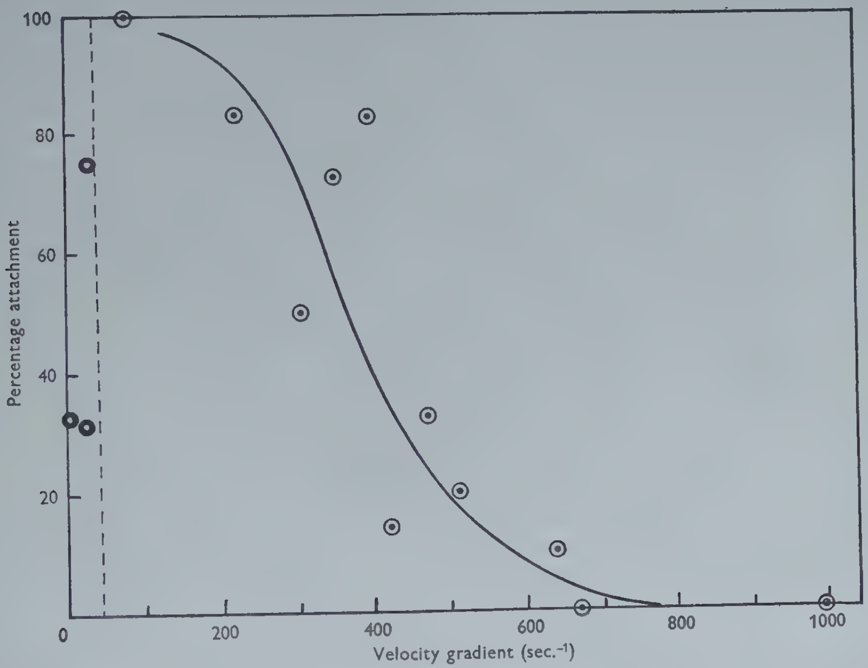

Fig. 4. Percentage attachment of cyprids of *Elminius modestus* plotted against velocity gradient in a tube of radius 0·091 cm. Open and solid symbols, and dotted lines have the same meaning as in Fig. 3.

so if the light was directed from one side of the tube. The cyprids observed walking in tubes appeared to be less influenced by a given gradient than those walking on a flat surface (see p. 580).

The observation that once the cyprid has obtained a hold, it can withstand and indeed walk in the face of strong currents, may be explained on the basis of the forces acting on the cypris.

The magnitude of the drag on a cyprid of *B. balanoides*, which measures about 1 mm. in length and 0·5 mm. in height, may be calculated approximately by considering simple models of similar size. If a short lamina 1 mm. long and 0·5 mm. high were lying in the plane of the current, the drag F on each side given by the Blasius formula $\approx 1{\cdot}3\,\rho^{\frac{1}{2}}U^{\frac{3}{2}}l^{\frac{1}{2}}\eta^{\frac{1}{2}}b$, where $\rho = 1$, $\eta = 0{\cdot}01$, $b = 0{\cdot}05$ and $l = 0{\cdot}10$. Thus for a mean current U of 50 cm./sec., at the level of the cyprid, corresponding roughly to a shear of over 1000 sec.$^{-1}$, the force acting on each side of the lamina will be approximately 0·7 dyne, making a total force of 1·4 dynes. If instead we consider the drag on a sphere of diameter 1 mm., Stokes (1850) formula, $F = 6\pi\eta rU$, gives approximately 0·5 dyne. The order of magnitude of the force required to hold the cyprid stationary in a very high velocity gradient of 1000 sec.$^{-1}$ will therefore be about 1 dyne. Indeed, since the cyprid is actually somewhat streamlined, the necessary force may well be rather less. If now we assume that the antennulary sucker is efficient, and acquires a suction of about 1 atmosphere, then the critical force to pull away a sucker measuring 40 μ in effective diameter will be $\pi r^2 P$, where $P = 1$ atmosphere $\approx 10^6$ dynes, $r = 2 \times 10^{-3}$ cm. Hence the combined suction of

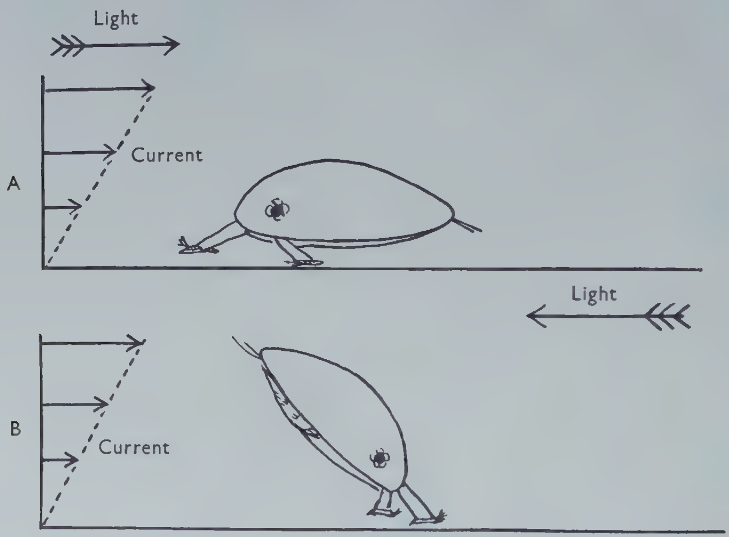

Fig. 5. Cypris larvae of *Elminius* crawling towards the light in a glass tube with surface velocity gradient exceeding 600 sec^{-1}. A, light and current from same direction, cyprid able to walk; B, light and current from opposite directions, cyprid lifted at hind end and often unable to continue without becoming detached.

two antennules ≈25 dynes. Thus, even allowing for some inefficiency in developing suction, the forces holding the antennules to the substratum appear to be adequate to withstand directly the drag caused by very high velocity gradients on the body of the cyprid. The actual diameter of the sucker is about 40–45 μ in *B. balanoides* and 20–25 μ in *E. modestus*.

ATTACHMENT IN LIGHT AND DARK

Since attachment in a water current appears to be preceded by rheotaxis the question arises as to the type of stimulus necessary for the rheotactic movements. It is known that fish maintain position in a current by responding to movements of the visual field (Lyon, 1904; Russell, 1938), though Gray (1937) has shown that blinded fish can respond to a limited extent to rotational currents. It appeared to be unlikely that cyprids use a visual mechanism, first, because the simple structure of the paired eyes would hardly allow image formation, and secondly, because the above experiments were performed in glass tubes placed at some distance from any object which could serve as a background. However, as the work of a number of observers has shown (Pomerat & Reiner, 1942; Weiss, 1947) light is undoubtedly an important factor affecting barnacle settlement.

Table 2. *Time of stay in tube* 1·13 *mm. radius, with shear* 142 *sec.*$^{-1}$

	Light (sec.)	Dark (sec.)
Inert	12·4	12·4
Living	30	12
	17	50
	14·5	16·5
	26	16·5
	16	13·5
	41·5	40
	45	55
	20·5	12
	16	50
	16	35
Mean	24·25	30·05

To test the effect of background on attachment, dark strips of paper were placed round the glass tube at intervals, and cyprids of *E. modestus* were introduced under a moderate gradient of 200 sec.$^{-1}$. The disposition of attached cyprids was noted. More cyprids were found nearer the entrance of the tube, as would be expected, since they sought to find a hold as soon as they were exposed to the current, but otherwise no significant pattern of attachment was obtained. The cyprids were found no more frequently in the light than in the dark sections, nor were the junctions of light and dark specially favoured.

Attachment in complete darkness was also examined. The rate of passage of an inert (dead) cyprid of *B. balanoides* was first determined under a velocity gradient of about 150 sec.$^{-1}$ by timing it between the 1 m. marks in the tube. Live cyprids of the same species were then introduced under artificial light one at a time, and the light immediately switched off. After a passage of time of about 1 sec. less than that required for the passage of a dead cyprid, the light was switched on again and the end of the tube carefully watched to see if the cyprid was emerging. If it was not, the tube was rapidly examined to find the cyprid, which was then followed down the tube and its total time of stay measured. After some experience it was found that attachment occurred regularly in the dark, and the period of darkness was extended beyond the time which would have been taken for an inert cyprid to pass through the tube. The results of a number of trials are given in Table 2, and show that the time of stay in the tube is not significantly influenced by light. Cyprids were also introduced into the tube in dim red light, to which many arthropods are insensitive, and were seen to swim against the flow and to attach exactly as in the light.

MECHANISM OF ATTACHMENT

The negative rheotaxy of the cyprid is worth commenting upon. It appears to be a response either to current variation near a solid boundary, or more probably to contact with it, and appears to lead almost immediately to attempts to make fast. Although attachment by the antennule cannot be seen in detail visually, certain inferences may be drawn as to the mechanism. From the velocity gradients at which attachment occurs fairly readily, viz. up to 150 sec.$^{-1}$ for *B. balanoides* and 250 sec.$^{-1}$ for *Elminius*, the distance at which the water velocity will equal the maximum rate at which the cyprid can swim may be calculated. For *B. balanoides* this will be $4{\cdot}7/150 \approx 0{\cdot}03$ cm. and for *Elminius* it will be approx. $4/250 \approx 0{\cdot}015$ cm.

The total height of the cyprids of these species when crawling flat on the substratum is about 0·06 and 0·03 cm. respectively. Hence maximum attachment occurs when the current at half the height of the cyprid from the surface has a velocity just equal to the cyprid's own maximum swimming velocity. It is a reasonable assumption therefore that on reaching the boundary they swim upstream and attach readily only if their own movements are capable of bringing them momentarily to rest. Since some attachment occurred at gradients exceeding those stated, either they can attach on occasion in spite of being carried downstream, or they can attain momentary bursts of speed in excess of those given in Table 1. As soon as attachment occurs the swimming movements presumably cease and the cyprid experiences drag due to the current. The shape of the substratum may considerably modify this drag, for if the cyprid is in a narrow tube or pit, only the upper surface will experience drag, whereas if attached to an edge almost the whole surface will be surrounded by a thin boundary layer and will experience a much greater pull. This may be the factor causing the apparently greater effect of current upon crawling on a flat surface than in a narrow tube (see p. 578) and may be one of the reasons for the cyprids' avoidance of sharp edges and their predilection for pits (Crisp & Barnes, 1954).

IS THE VELOCITY GRADIENT AN ADEQUATE DESCRIPTION OF CONDITIONS OF ATTACHMENT?

It is of interest to compare these results with those reported by Walton Smith (1946), who correlated settlement (as distinct from attachment in our sense) with the water velocity measured at some distance from the settling surface. His first method of obtaining a relative movement of substratum and water, that of rotating a large disk in the sea, is not readily amenable to hydrodynamic analysis. Cochran (1934), however, gives a solution for the drag on the surface of a rotating disk of infinite size as

$$\left(\frac{\partial v}{\partial z}\right)_0 = \rho^{\frac{1}{2}}\eta^{-\frac{1}{2}}\omega^{\frac{3}{2}}rg', \tag{11}$$

where ω is the angular velocity and r the radius, g' a constant $\approx 0{\cdot}616$, $(\partial v/\partial z)_0$ is the gradient of tangential velocity along an axis normal to the disk; ρ is the density, and η the viscosity of the fluid.

This solution applies strictly to a large smooth disk rotating in a perfect plane.

Walton Smith's second method, that of using a series of glass tubes, is somewhat more suitable for analysis. Provided the tubes were long and that the turbulence developed at the junctions did not seriously upset the flow, equations (7) and (8) above (p. 572) are exact solutions. Table 3 shows the results given in his tables 1 and 2, recalculated on a basis of rate of shear instead of the radial velocity of the disk or the mean velocity in the tube. It will be seen that for the tubes a limiting velocity gradient of about 100 sec.$^{-1}$ fits the observations for most of the barnacles, *B. amphitrite* and *B. eburneus* being rather more sensitive and *B. improvisus* less sensitive to shear. These values are slightly lower than the highest gradients which still gave attachment of *E. modestus* and *B. balanoides*. Possibly this difference is because in Walton Smith's experiments only final settlements were observed, whereas in the experiments on *B. balanoides* and *E. modestus* initial attachment, however temporary, was included. If such temporary attachments took place in the narrower sections of the graded tubes they might well have been missed, since the larvae on swimming off would immediately be carried to the wider tubes where they would have a longer time in which to settle. A comparison on the basis of mean flow velocity gives no semblance of agreement between Walton Smith's observations and those described here. Thus the limiting average flow velocity in Walton Smith's tubes, which were of large diameter, was about 1·0 mile/hr., which is equivalent to 45 cm./sec. In a smaller tube of 6·4 mm. diameter the limiting average flow velocity for *B. balanoides* now proves to be about 20 cm./sec., and in a tube of 2·3 mm. diameter it is only 12 cm./sec. *E. modestus*, passed through an even narrower tube of diameter less than a millimetre, gives limiting flow velocities of only 6 cm./sec. Yet all sets of results agree well in their orders of magnitude if expressed in terms of limiting velocity gradient. Walton Smith's findings give the appearance of consistency on a basis of average flow velocity only because he did not use a wide range of tube diameters in his experiments.

The calculations of shear for the rotating disk method must be treated with some reserve because of assumptions implicit in Cochran's (1934) analysis, but the limiting values are so high that some explanation seems to be needed to account for the lack of agreement with the results from the tube experiments. Several possible reasons may be advanced for the discrepancy. First, the disk may have rotated unevenly, giving rise to areas of high and low rates of shear, or its surface may have been itself uneven and so allowed lower gradients to exist in small depressions. Secondly, the experiments differed from those in tubes in one fundamental respect, namely that the one experimental surface was exposed to variable shear, the gradient rising sharply from a zero value at the centre to a high value at the edge. Attachment

Table 3. *Recalculation of results taken from Walton Smith* (1946) *in terms of velocity gradient (reciprocal seconds) at surface of tube instead of mean velocity of flow down the tube, which is given in brackets as miles per hour**

(Only values to the right of the firm line were suitable for barnacle settlement.)

Period and place	Rate of flow q (ml./sec.)	Surface velocity gradient $(\partial v/\partial r)_0$ in glass tubes of radius r calculated from $(\partial v/\partial r)_0 = 4q/\pi r^3$				
		r=0·7 cm.	r=1·1 cm.	r=1·4 cm.	r=1·85 cm.	r=2·5 cm.
17. xi. 43 to 27. xi. 43, Miami Beach	217–267	807–992 (3·0–3·7)	208–256 (1·2–1·6)	102–125 (0·8–1·0)	43·7–54·0 (0·5–0·6)	17·8–21·9 (0·2–0·3)
					←*B. amphitrite*→	
20. v. 44 to 12. vii. 44, Kure Beach	350	1300 (5·3)	336 (2·3)	164 (1·4)	71 (0·8)	28·7 (0·4)
					←*Balanus* sp.→	
12. vii. 44 to 18. ix. 44 Kure Beach	212	760 (3·2)	196 (1·3)	96 (0·8)	41 (0·5)	16·8 (0·2)
					←*Balanus* sp.→	
18. ix. 44 to 25. xi. 44, Kure Beach	180	670 (2·7)	173 (1·1)	84 (0·7)	36·3 (0·4)	14·8 (0·2)
				←*B. improvisus*→	←*B. eburneus*→	

Recalculation of results for rotating disk in terms of velocity gradient $(\partial v/\partial z)_0$ at disk surface instead of tangential velocity of a point on the disk (ωr)

Disk	Speed of rotation (radians/sec.) (ω)	Maximum attachment radius (cm.) (r)	Velocity gradient (sec.$^{-1}$) $(\partial v/\partial z)_0$	Tangential velocity (knots) (ωr)
1B	56·5	0·95	2460	1·1
4A	20·1	2·55	1420	1·0
6A	14·0	3·20	1070	0·9
7A	6·3	10·2	1000	1·3

* The calculations of table 2 (Walton Smith, 1946) appear to be in miles per hour, and not in knots as stated in his table.

where the rate of shear was low might well have been followed by migration of the cyprids outwards. Since the rotation of the disk produces an axial stream flowing towards the centre, cyprids would be drawn into contact with the region of low gradient initially, would attach without difficulty, and might then migrate as usual by walking. On this interpretation the gradients given by this experiment are the maximum gradients across which cyprids can walk once they have successfully attached.

While no direct evidence can be presented that fixation is possible under conditions of flow more rigorous than those which limit attachment, the migrating larva has been observed to withstand gradients greatly in excess of those which prevent attachment, and it is reasonable to suppose that it could also metamorphose under such conditions.

It is not claimed that the velocity gradient at the boundary automatically controls the possibility of attachment, for one assumption has been made which can only partially be true. This is that the cyprid takes up the velocity of the water in which it finds itself, or alternatively that it can swim at a certain speed against the velocity of the water in this layer. In fact, however, if it swims rapidly across a velocity gradient its own inertia will tend to prevent its slowing down to the speed of the layers near the boundary. It may therefore need to swim along the boundary for a short time while it slows down. In other words, the behaviour of the cyprid at the moment of attachment is very important.

The magnitude of this inertia effect may be roughly estimated by considering instead of the cyprid a moving sphere of approximately equivalent mass and viscous drag, to which Stokes's (1850) formula is applicable. This gives the drag due to viscous forces as

$$F = -6\pi\eta a V,$$

where V is the velocity of the sphere relative to the medium, a its radius, and η the Newtonian viscosity of the medium.

The deceleration $-dV/dt$ of such a body of density ρ moving freely will therefore be

$$-\frac{dV}{dt} = \frac{6\pi\eta a V}{\frac{4}{3}\pi a^3 \rho} = \frac{9\eta}{2a^2\rho} V.$$

Putting $\frac{9\eta}{2a^2\rho} = \lambda$,

$$-\frac{d \log_e V}{dt} = \lambda,$$

hence the subsequent motion is given by

$$V = V_0 e^{-\lambda t},$$

where V_0 is the velocity at zero time, and e is the base of natural logarithms.

Now we shall assume that the body is caused to move with a velocity V_y across a velocity gradient $(\partial v/\partial y)$, that is along the y axis of Fig. 6. Then if V remains the velocity relative to the medium measured along the x axis, this will be given by

$$V = V_x - v_m,$$

where V_x is the velocity of the body along the direction Ox, and v_m that of the layer of fluid in which it is moving. Then, ignoring viscous forces operating along the x axis,

$$\frac{dV}{dt} = -V_y\left(\frac{\partial v}{\partial y}\right).$$

Bringing in viscous forces the equation of motion becomes

$$-\frac{dV}{dt} = V_y\left(\frac{\partial v}{\partial y}\right) + \lambda V.$$

Putting $\alpha = V_y\left(\frac{\partial v}{\partial y}\right)\Big/\lambda$, the solution becomes

$$V = (V_0 + \alpha)e^{-\lambda t} - \alpha.$$

When $V_0 = 0$, this equation becomes

$$V = \alpha(e^{-\lambda t} - 1),$$

V being a direct measure of the discrepancy between the motion of the body and that of the fluid after the body has moved across the velocity gradient for a time t.

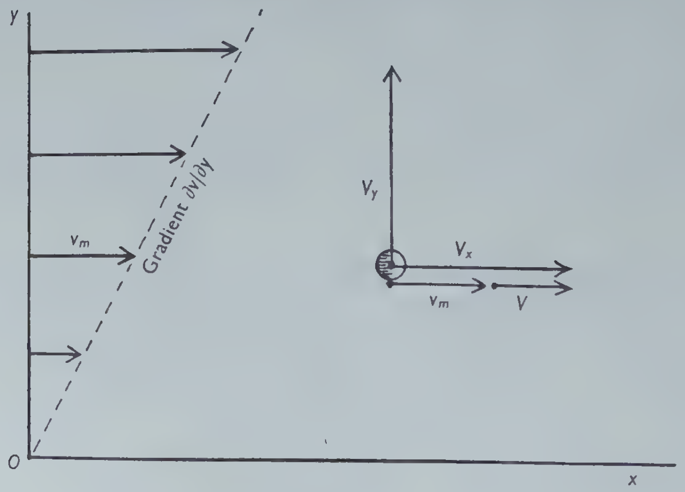

Fig. 6.

Since the term in brackets is negative, a movement away from the fixed boundary (V_y positive) causes a displacement of the body against the stream (V negative), but movements towards the boundary (V_y negative) cause the body to be carried along the x-axis faster than the stream. Thus an animal swimming rapidly towards a surface will tend to be carried past a given point on the surface faster than would have been the case if it approached more slowly. Nevertheless, if λ is large these displacements rapidly disappear, the velocity relative to the fluid (V) becoming zero in a very short time. The equations given above allow the magnitude of V for reasonable values of other constants to be calculated. Thus taking $a = 0{\cdot}03$ cm., $\rho = 1$, and

$\eta = 0.01$ poise, the time constant $\lambda \approx 50$ and the half life of any displacement velocity will be less than 0·015 sec. The animal need therefore remain at the boundary for only about 0·025 sec. to acquire virtually the same velocity as the water.

ORIENTATION OF CYPRIDS METAMORPHOSING IN WATER CURRENTS

Knight-Jones (1953) has shown that cyprids of several species of barnacle do not metamorphose readily unless other previously settled individuals of the same species are present on the substratum. Glass tubes already bearing such individuals were not available, hence for experiments on metamorphosis it was necessary to use bakelite plates with previously settled individuals occupying small pits drilled in the surface. These were placed in a shallow Bakelite trough within which a continuous circuit of water was maintained as described above. About 15–20 cyprids were introduced in each experiment, and the flow of water over the plate was varied in direction and in magnitude from one experiment to another. To avoid any orientation due to differential lighting (Barnes, Crisp & Powell, 1951) the experiments were carried out in a dark room. After 24 hr. the majority of the cyprids had usually settled and the orientation of each was measured. Fig. 7 shows as a histogram the orientation of 252 individuals. There appears to be a small but significant tendency for settlement with the posterior end pointing *upstream*, an orientation shown to be difficult with high velocity gradients. The velocity gradients obtained in this

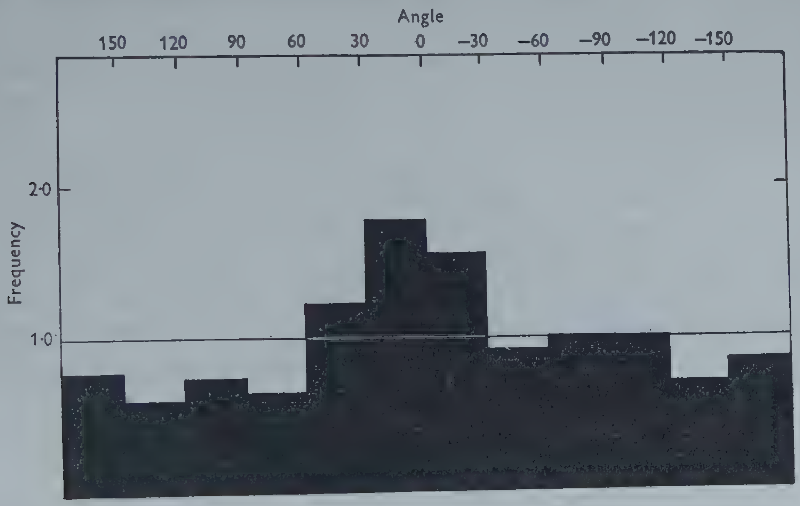

Fig. 7. Orientation histogram of 252 individuals settling in the dark on a plane surface over which a steady water current was passing. An orientation with the anterior end pointing downstream is taken as 0° and with the anterior end upstream as 180°. Orientations in a clockwise (anticlockwise) direction are shown as positive (negative) angles of appropriate magnitude. Frequencies are given as the ratio of number observed in each 30 degree range to the random expectation of 21 (252 ÷ 12). The line of unit frequency is drawn to indicate the expected value were no orientational forces at work. The χ^2 test indicates that the peak at zero angle is significant at $P \approx 0.01$.

apparatus, however, were relatively small, ranging from 10 to 60 sec.$^{-1}$. The orientation induced was so slight that it was not possible to determine whether there was any relation between the degree of orientation and the magnitude of the velocity gradient. A few experiments carried out under directional lighting showed that the current had no appreciable effect on the strong orientation to light.

Cyprids settling in the current were observed to walk in many directions, not in any way related to that of the current, and to explore the substratum in a way similar to that shown in the absence of current. If the current was reversed while a cyprid was walking at right angles to it, the cyprid momentarily swung round to face the new direction of thrust, but then twisted back to restore itself to its original position so that it resumed its progress in the same direction as before.

Evidently the orientating influence of water currents is much less than that of light (Barnes *et al.* 1951) and of surface contour (Crisp & Barnes, 1954). However, it should be emphasized that the current would orientate a passive cyprid so that its posterior end pointed downstream. Hence the observed orientation (or indeed even an apparently random orientation) reveals an active opposition to the mechanical forces caused by the current. It seems likely that this weak orientation to current is occasioned by a tendency to over-compensate for the twist exerted by the current rather than to the detection of the current followed by a deliberate orientation to it. In this respect it differs from the reaction to light and surface contour which are genuine orientation reactions.

The effect of water current on settlement at high velocity gradients could not be investigated since gradients exceeding 100 sec.$^{-1}$ could not be readily maintained in this apparatus. It was established, however, that settlement occurs readily in *B. balanoides* and in *B. crenatus* at gradients of 40–60 sec.$^{-1}$.

INFLUENCE OF WATER MOVEMENT ON DISTRIBUTION AND SURVIVAL

The attachment response evoked when the cyprid is carried against objects lying in a water current is of obvious value in allowing settlement to take place on wave-washed shores or on objects lying in the path of strong tidal currents. The magnitude of the velocity gradient under which attachment can take place is high; a gradient of 100 sec.$^{-1}$, under which attachment readily occurs, corresponds to a water velocity of 1 m./sec. in a laminar régime at a distance of only 1 cm. from the substratum. Evidently the development of this mechanism reflects the great survival value of attachment under rigorous conditions. It would seem reasonable, in view of the small areas of suitable substrata that exist in the enormous volumes of water where cyprids are dispersed, that no opportunity for attachment should be missed. It is therefore at first sight surprising to find that *B. balanoides* and to a lesser extent *E. modestus* do not attach as readily to surfaces under still conditions as when they are exposed to some degree of water movement. The adaptive value of this behaviour, however, is probably its selectivity, for it would encourage settlement in exposed positions at the expense of those where the water was stagnant, and its operation would account for the observations of Southward (1953) that greater settlements of *B. balanoides* occur in areas of moderate exposure than in shelter. Barnes & Powell (1953) also have emphasized the importance of water movement, and similarly accounted for the intertidal distribution of this species, attributing its absence below low-water mark to the fact that there is no periodic washing by waves. Pyefinch (1948) refers to the clinging response of *B. balanoides* evoked by water currents, but states that it is not shown by *B. crenatus*. The difference is, I believe, rather one of degree, since I have observed *B. crenatus* attaching and metamorphosing in weak currents. However, if the response to water movement differed from one species to another, it would provide a possible mechanism to account for the distribution of barnacles in relation to wave exposure. Thus some species, notably *Chthamalus stellatus*, favour very exposed habitats (Hatton & Fischer-Piette, 1932; Moore & Kitching, 1939), while others such as *E. modestus* are found mainly in shelter (Moore, 1944).

Moreover, the stimulus to settle where there is a current of water may have a general advantage, for it is a matter of common observation that exposed headlands and narrow passages carrying strong currents, where the water is rapidly replenished, often support rich populations with individuals of large size. These are clearly the most advantageous for a sessile form to colonize. For example, Fox & Coe (1943) observed greatest growth of *Mytilus calafornianus* on wave-washed shores, while Bishop (1954) has found individuals of *E. modestus*, which normally grows to about 8 mm. diameter, to reach over twice this size on bridges spanning the narrow drowned river valleys of N. Finisterre, where the large tidal range produces exceptionally fast currents. The advantageous effect of water movement can also operate very locally, for Hentschel (1923) records the greatest barnacle growth on the most exposed parts of ships, while I have frequently observed that *M. edulis* grows most rapidly at the exposed corners of pontoons, piers, etc. The most advantageous positions for barnacles to settle are therefore those where the water movement is as great as they can tolerate. Stagnant water is disadvantageous, not only because it limits the food supply, but also because silting up is a serious danger to young spat (Coe & Allen, 1937; Fuller, 1946).

A further advantage would accrue if the position of settlement were such that the current were usually directed into the cirral net when the barnacle was fishing (Moore, 1935; Crisp, 1953*b*). It is therefore interesting to note that the observed tendency for the posterior end to face the current would, after metamorphosis, achieve this result.

Water currents produced by the movement of ships will limit settlement on hulls, in a similar way as on natural substrata. The majority of species fouling ships are sublittoral, and therefore probably adapted to settle under very small or even zero gradient. Ships will therefore acquire immunity from settlement only when in motion, as has been found in practice (Neu, 1932; Hentschel, 1934). When a vessel is under way, the velocity gradients will vary according to position, being greatest at the front, especially in vessels of full form. This is true, whether the motion is turbulent or otherwise. Fouling, where limited by the velocity gradient, would therefore be expected to start at some point along the length of the ship and to be particularly in evidence at the after end, where relatively stationary pockets of water develop. This has been noted on slow moving ferries, and on ships moored for long periods in a tideway.

In practice, the flow past a ship is invariably turbulent, since the critical Reynolds number is exceeded even at very moderate speeds. The velocity gradient at the surface will be correspondingly large and an average figure can be calculated on the basis of Froude's equation. Expressing $(\partial v/\partial y)_0$ and η in metric units, and f in dynes per cm.^{2} of wetted surface, we obtain for a ship of 400 ft. length with a smooth painted surface (Eqn. 6)

$$(\partial v/\partial y)_0 = 342\ U^{1\cdot86},$$

where U is expressed in knots. Hence at 1 knot the average shear will be 342 sec.$^{-1}$, and will increase with velocity, becoming 1250 at 2 knots, and 24,800 at 10 knots. Hence, if we accept a figure of 500 sec.$^{-1}$ as the absolute limit for barnacle attachment, no barnacle settlement would be possible at speeds exceeding 1–2 knots, provided that the gradient were uniform over the whole ship. In fact, considerable variation will occur; not only will the after end as a whole experience lower shear, but lapped plates and rivets will cause local eddies where the velocity gradient may be reduced, so that regions of low shear may allow settlement even at velocities slightly higher than 2 knots. It is important, however, to notice from Froude's experiments that the gradient increases very sharply with velocity, but that it is not very materially influenced by the size of the object above a certain minimum. Thus for a ship of only 100 ft. the constant f increases only by 20%. Hence, while local variations in velocity gradient may allow settlement to occur at velocities slightly exceeding 1 or 2 knots, at water speeds exceeding, say, 5 knots, settlement would cease altogether. The sharp increase in shear with increase in velocity, and its relative independence of the size of the object, has undoubtedly given rise to the view that the velocity itself, rather than velocity gradient, controls settlement. For empirical purposes, moreover, it is justifiable to assume that a large flat object exposed to a relative motion of 3 knots or more parallel to its surface will not become fouled.

A factor which has not been considered in this paper, but which is certainly of great importance in practice is the influence of suspended matter on the settlement of larvae. Particles, if of size comparable with the larvae, might on account of their density and consequent inertia very materially accentuate the inhibition of settlement by water currents. The scouring action of certain sea areas was in the past a well known remedy for accumulated fouling. The effect, too, can be seen on rocks standing on a sandy shore. These are often well covered in barnacles, except for a bare zone some 2 or 3 ft. from the sea bed, caused by the abrasion of sand particles lifted by the waves.

SUMMARY

1. The velocity gradient of the fluid close to the solid boundary is the most appropriate description of the conditions of water flow which affect the attachment of the larvae of sessile forms to solid objects. The nominal speed of the water move-

ment past the object is of importance only in so far as it influences the velocity gradient in the boundary layer.

2. Experiments in glass tubes on cypris larvae of *Elminius modestus* and *Balanus balanoides* show that moderate velocity gradients exceeding 500 sec.^{-1} sweep the cyprids past the surface before they can attach. For large objects exposed to turbulent flow, the critical velocity gradient corresponds very approximately to a flow of 1–2 knots.

3. Attachment under conditions of water flow is accompanied by negative rheotaxy, and can occur equally in the light and in the dark.

4. Maximum attachment occurs at or below velocity gradients just great enough for the cyprid to be able to maintain position by swimming along the surface against the current.

5. Once the cyprid has attached it cannot be pulled off the surface even by gradients greatly in excess of those which prevent attachment.

6. Cyprids can migrate in all directions when exposed to moderate gradients, but they do so only with difficulty when the velocity gradient is high, particularly if the water is flowing in the same direction as that in which the cyprids are walking. The direction in which the cyprid migrates is altered only momentarily by changes in the direction of the current, the animal actively resisting the redistribution of forces acting on it.

7. No direct evidence is given in this paper on the influence of water currents on fixation, but a critical comparison with other published work suggests that fixation can occur in places where the velocity gradients are greater than those which limit attachment, provided the cypris is able to migrate there after attachment.

8. Moderate velocity gradients have little effect on the orientation at metamorphosis. Individuals tend to settle with the anterior end pointing downstream rather than in any other position. This orientation is the opposite from that which would be expected if the cyprid were passively orientated by the water current, but is likely to make subsequent feeding more efficient.

9. The ability to attach under conditions of water flow, and the tendency not to attach under stagnant conditions, may have an important influence on the animals' distribution and survival.

10. Solid particles in suspension may profoundly influence the behaviour, hence the results given in this paper may not be relevant to conditions where scouring takes place.

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THE WETTING OF INSECT CUTICLES BY WATER

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INTRODUCTION

The wetting properties of insect cuticles are of fundamental importance in insecticidal spray development and application. For this reason there is an extensive literature concerned with the qualitative and quantitative estimation of the adherence and spreading of various liquids on cuticles (Ebeling, 1939; Hoskins, 1940; O'Kane, Westgate, Glover & Lowry, 1930; O'Kane, Westgate & Glover, 1932, 1937; Stellwaag, 1924, etc.). But, as Richards (1951) has pointed out, the picture presented by this work is a complex one, and it is seldom possible to reach any valid conclusions as to the factors determining the results obtained. Quantitative information has in all cases been derived from measurement of the angles of contact of test liquids on the insect surfaces, and the position is complicated by the variety of the methods used. Some of these methods, by their failure to take precautions against contamination, must in any case be regarded as of doubtful value. Even the most extensive and recent work, by Pal (1951), suffers from this latter defect; it is also far more descriptive than analytical in character.

The information obtained from the field of applied entomology is supplemented by some qualitative data from studies on aquatic insects (Brocher, 1910; Miall, 1891, 1892, 1895; Crisp & Thorpe, 1948). In these animals, surface properties are often of great ecological significance.

From the mass of data available, it is apparent that terrestrial insects are generally hydrophobic and lipophilic, while aquatic species are often (but not always) hydrophilic and, after drying, lipophilic as well (Richards, 1951). The importance of surface roughness as a factor modifying the contact angles has been noted by Hoskins (1940) and by Pal (1951), and studied in some detail for an aquatic species with a 'plastron' by Crisp & Thorpe (1948). The existence of variations in wetting properties that can be correlated with processes of cuticle secretion has been demonstrated by Wigglesworth (1947, 1948), by Lees (1947) (in ticks), and confirmed by Pal (1951). There has, however, been no systematic analysis of the wetting properties of insects comparable to that carried out by Fogg (1947, 1948) for plants, and the present work was undertaken in an attempt to remedy this deficiency.

THE THEORY OF THE CONTACT ANGLE

All serious work on the wetting of solids by liquids has been expressed quantitatively in terms of the contact angle. Since the biological literature on this subject is particularly misleading the physical factors involved in such measurements must be briefly considered.

When a liquid rests upon a solid, or a solid dips into a liquid, the liquid-air interface usually meets the solid-air interface at a definite angle which is a physical constant for the substances concerned. This angle, measured in the liquid, is the contact angle.

There is still some controversy over the interpretation of this phenomenon, and it has even been stated recently that 'there is no satisfactory explanation of the contact angle' (Yarnold & Mason, 1949). Most authors, however, consider that the contact angle depends on the adhesion forces between liquid and solid, and Adam (1941) gives the equations:

$$\cos\theta = \frac{(y_{sa} - y_{sl})}{y_{la}}$$

and

$$w_{sl} = y_{la}(1 + \cos\theta),$$

where w_{sl} is the work of adhesion between solid and liquid, y_{sa}, y_{sl} and y_{la} are the surface tensions between solid and air, solid and liquid, and liquid and air, respectively, and θ is the contact angle. These equations are not accepted by Yarnold & Mason (1949), who consider the determining factor is not the direct attraction between solid and liquid, but the extent to which the solid surface is covered by an adsorbed film of molecules of the liquid before the main liquid mass reaches it.

In practice, most solids and liquids show pronounced hysteresis of the contact angle, the angle observed when the liquid advances over the solid (advancing contact angle) being larger than the retreating contact angle, observed when it withdraws. Again there is no satisfactory general theory to account for this phenomenon, which in different instances may depend on adsorption (Cassie, 1948), micro-roughness (Shuttleworth & Bailey, 1948), or removal of 'contaminant' (see Adam, 1948, for review).

There is no doubt that the contact angles of a solid surface depend upon the most superficial layer of molecules, and, with long chain organic acids, polar groups which have no influence on the contact angle may still be sufficiently near the surface to act as catalysts (Adam, Morrell & Norrish, 1925). Langmuir (1938) has shown that oleic acid monolayers deposited on a clean glass plate can raise its advancing contact angle against water from 0 to 86°. Blodgett (1934) has shown that calcium stearate monolayers on glass have large advancing angles against water when orientated with methyl groups outwards, but are readily wetted when reversed. These observations are of great importance, since they indicate that the surface properties of a solid may depend on layers so thin as to be undetectable by most other methods. They also demonstrate the critical importance of avoiding contamination of the solid surface; even exposure to the atmosphere for any length of time is liable to lead to the deposition of a lipid film, after which quite erroneous results will be obtained.

Another factor which exerts a profound influence on the contact angle is surface roughness. This acts to magnify the extent to which the angle differs from 90° (Wenzel, 1936). When the true advancing angle is above 90°, however, air is trapped in the hollows of a rough surface and this may lead to an enormous apparent contact angles (Cassie & Baxter, 1944). With spaced fibres orientated parallel to the interface it is theoretically possible to obtain large apparent angles as long as the true angle exceeds 0°, and with a true angle of 100° apparent values of over 160° are normal. Failure to appreciate the great influence of roughness is probably the cause of the diverse values for the advancing contact angle of paraffin wax against water that appear in the literature. When test surfaces of this substance are obtained by solidification in air angles of up to 110° may be observed, but after flattening by pressure on a glass plate the angle falls to 97° (Baxter, 1948). Conversely, roughness will produce a lowering of the observed angle if the true angle is less than 90°, and trapped air does not make up more than a small proportion of the interface.

The third major factor which exerts a modifying influence on the contact angles is the relationship between the solid surface and the test liquid prior to measurement. It is generally found that soaking a solid in a liquid for any length of time results in a large reduction in the contact angles observed (Nietz, 1928; Yarnold & Mason, 1949). In some cases this may be due to the replacement of an adsorbed air film by one of the liquid. Recently, however, Rideal & Tadayon (1954) have shown that the incorporation of small amounts of stearic acid in paraffin wax enormously increases the extent to which the advancing contact angle of the wax against water is lowered by soaking. With 9% of the polar material, for example, the angle fell from 110° to below 80° in 5 hr. In this case, therefore, the lowering in angle is probably due to a re-orientation of the polar molecules at the surface in response to the attraction of the water.

Summarizing the above information, it is evident that the deposition of foreign matter on the surface in minute quantities, the presence of roughness and the previous contact of the surface with liquids may all lead to the measurement of contact angles that bear little relation to the theoretical values expected on the grounds of chemical composition. Any contamination of the surface of the test liquid, by altering its surface tension, will also lead to considerable errors.

METHODS

Previous work on the wetting properties of insect cuticles has nearly all been based upon the measurement of contact angles at the margins of small drops of test liquid deposited on the insect surface. In all these cases (except the work of Beament, 1945*a*) no attempt was made to prevent the contamination of the droplet surface film. Since Beament (1945*b*) has shown that many cuticles contain materials liable to spread in a water surface even at room temperature the accuracy of these results is open to question. The observation of Pal (1951) that 'in general insects with a low melting-point cuticular wax show smaller contact angles than those with a hard wax' may almost certainly be attributed to the contamination of his droplet surface films. He cites the cockroach as an example of the insects with a low melting-point wax and low angles; in this animal it is well known that drops of water deposited on the surface are almost immediately covered by a grease film (Ramsay, 1935). Contact angle measurements made by these droplet methods are thus liable to a variable and uncontrollable source of error, and while the results are of value as an empirical assay of spray droplet behaviour, they cannot be used to give more than qualitative information about cuticle structure.

For this reason, an entirely different method has been used in the present work. It is based on the 'inclined plate' apparatus of Adam & Jessop (1925), a method previously used for biological material by Stellwaag (1924), although without surface cleaning. The specimens were usually killed before examination by exposure to cyanide or hydrogen sulphide gas; these agents were considered unlikely to affect the cuticle. The animals were then attached to clean glass rods by paraffin wax or cellulose paint, the attachment being made at a point as distant as possible from the region whose surface properties were to be examined. The rods were then mounted in a specially constructed apparatus (see Appendix), which allowed them to be tilted to any angle and moved slowly vertically and horizontally.

Tap water was in all cases used as the test liquid, and the water surface was contained in a waxed polythene vessel. A tube from the tap led to the base of the vessel so that the water could be run in from below, and the surplus water overflowed around the rim, thereby maintaining a clean surface. Tests with surfaces dusted with *Lycopodium* powder showed the efficiency of this cleaning method to be very great, and it was certainly preferable to the use of paraffin-waxed barriers.

Two methods of measurement of the contact angles were possible using this apparatus. One of these, the true 'inclined plate' method, was used where large areas of fairly plane surface were available. In this method, the inclination of the specimen to the water surface is adjusted until a position is found in which, when the specimen is lowered into the water by the vertical slow motion, the water surface remains undistorted right up to the point of contact. The inclination of the specimen to the water surface is then equal to the advancing contact angle, which may be read off a scale, the scale reading when the insect surface is vertical having been previously noted. The retreating angle is then found by a similar process of adjustment.

Where the surfaces available were very small, this method proved difficult to operate. Instead, therefore, of adjusting the inclination to give a horizontal water surface at the point of contact, the specimens were set so that the meniscus curved slightly upwards. A horizontal microscope, mounted level with the water surface, allowed this meniscus to be observed, and the tangents to the solid and liquid surfaces were drawn by means of a camera lucida attachment. The advancing angles having been observed by lowering the specimens slowly by the vertical motion, the retreating angles were found by a similar process of adjustment when the specimens were raised. It was unfortunately impracticable to record the contact angles while the specimens were actually in motion; the values quoted are all 'equilibrium' contact angles measured as soon as possible after the motion had stopped and the meniscus come to rest. It is recognized that this is less desirable on theoretical grounds (Yarnold & Mason, 1949); none the less, the inaccuracies introduced by this process are no greater than those inherent in the material. For each specimen, the advancing and retreating angles were determined at least 5 times, usually on slightly different regions of the surface, and means taken. After each series the surface of the water was thoroughly cleaned, and a slow rate of flow was usually maintained throughout the process of measurement, since this changed the surface continually without producing turbulence.

The results obtained from these two methods were in excellent agreement, and repeated measurements on the same specimen or on different individuals of the same species and instar also yielded consistent results (Table 1). Much of the observed variation is considered to be due to genuine differences in the surfaces studied. The mean angles given in this paper for species are always expressed to the nearest degree, and a range of variation of five degrees on either side of this value is regarded as normal. When it is considered that the most precise physical methods have given values of 97–113° for the advancing contact angle of paraffin wax, it is clearly improbable that the far more heterogeneous surfaces of insects will give results of any greater precision.

Table 1. *Consistency of results*

(Date for *Tenebrio molitor* pupae, less than 10 hr. old.)

Specimens	Advancing angles	Retreating angles
Successive determinations		
1	111, 113, 111, 114, 118, 113	97, 92, 97, 88, 90, 92
2	112, 111, 115, 110, 107, 110	93, 88, 92, 92, 90, 96
3	111, 112, 101, 105, 107, 113	93, 82, 89, 90, 90, 86
4	110, 116, 114, 110, 120, 110	86, 82, 90, 91, 93, 91
Mean values, 12 other specimens	113, 108, 117, 108, 108, 106, 105, 111, 112, 112, 106, 109	94, 92, 95, 102, 99, 95 91, 91, 88, 92, 95, 87

THE CONTACT ANGLES OF INSECTS AGAINST WATER

The advancing and retreating contact angles for a wide range of insects are included in Table 2, and demonstrate very clearly the considerable variation that exists within the group. Four main subgroups have been recognized in the table, and these will be briefly discussed.

The first group contains both terrestrial and aquatic species whose surfaces are very rough or covered with a hair pile. They all have advancing angles that exceed 150°, and approach 180° in some cases, and their retreating angles are also large.

The second group contains only smooth-surfaced terrestrial species, whose advancing angles are in the region of 100–110°, and whose retreating angles are about 80–90°. They thus approximate in their wetting properties to paraffin wax.

The third group contains smooth-surfaced aquatic species, whose advancing angles are a little lower than those of group 2, and whose retreating angles are often considerably lower. It is interesting that these species all leave the water at some time in their lives, and are surface breathing, whereas the next group from which they differ markedly in surface properties are all totally submerged.

The fourth group, the totally submerged aquatic species with rough surfaces, have very low advancing and retreating angles. When removed from the water, there is usually a film of water retained over the surfaces, and a finite advancing angle is not observed until the animals have dried.

Two important conclusions can be drawn from this series of results. In the first place, it is clear that the inter-specific differences that exist largely reflect differences in surface roughness and habitat. In the second place, it is evident that there may be large differences in wetting properties between different regions of the surface of a single species, and these differences also are correlated with variations in roughness. A great deal of the observed variation could therefore be accounted for in terms of surface contour and the effect of prolonged immersion in water, assuming

Table 2. *The water contact angles of insects*

Group	Species	Advancing angle	Retreating angle	Surface	Habitat
1	*Calliphora erythrocephala* (adult)	175	155	Hairs	Terrestrial
	Coenagrion puellam (adult)	175	170	Hairs	Terrestrial
	Notonecta glauca (fore-wings, upper surface)	c. 180	c. 180	Hairs	Aquatic
	Rhodnius prolixus (5th, dorsal abdomen)	165	110	Rough	Terrestrial
	Tenebrio molitor (mature pupa, prothorax)	155	110	Rough	Terrestrial
	Locusta migratoria (prothorax, flanks)	128	104	Rough	Terrestrial
2	*L. migratoria* (fore-wings)	107	89	Smooth	Terrestrial
	Periplaneta americana (elytra)	106	91	Smooth	Terrestrial
	Tenebrio molitor				
	Adult; elytra	107	92	Smooth	Normally stored products*
	Larva; dorsal	107	82	Smooth	
	Pupa; first day	109	91	Smooth	
	Agonum obscurum (Col. Carabidae)	96	80	Smooth	Terrestrial
	A. fuliginosum	102	89	Smooth	Terrestrial
	A. viduum	101	84	Smooth	Terrestrial
	A. thoreyi	104	89	Smooth	Terrestrial
	Dysdercus cingulatus (nymph; dorsal surface)	98	84	Smooth	Culture
	Gyrinus marinus (elytra)	105	90	Smooth	Water surfaces
3	*Notonecta glauca* (prothorax)	98	70	Smooth	Aquatic
	Naucoris cimecoides (prothorax)	90	56	Smooth	Aquatic
	Dytiscus marginalis (elytra)	90	10	Ribbed	Aquatic
	Hydrobius sp.	87	50–0	Smooth	Aquatic
4	*Ranatra linearis* (elytra)	62	0	Rough	Aquatic
	Nepa cinerea	59	0	Rough	Aquatic
	Anax imperator (nymph)	45	0	Rough	Aquatic
	Coenagrion puellam (nymph)	35	0	Rough	Aquatic

* These values from clean specimens

that in respect to these factors insect surfaces behave similarly to the surfaces investigated by physicists. A more detailed study has therefore been made of them, while observations have also been made on the effect of contamination of the cuticle, and on changes in surface properties in relation to processes of cuticle secrction.

THE EFFECT OF CONTAMINATION ON THE CONTACT ANGLE

The wetting properties of any surface may be enormously modified by the presence of traces of foreign material. In nature it is evident that abundant opportunities for such contamination exist. None the less, the biological literature contains no discussion of the possible importance of this factor. A few actual instances have been noted; Pal (1951) records, for example, that he was unable to obtain consistent figures for the contact angles of *Musca* larvae. When it is considered that these larvae spend their lives tunnelling through meat, this observation is hardly surprising. Hoskins (1940) notes that the advancing contact angle of *Psylla pyrisuga* nymphs may be as low as 40° (whereas aphids in general have angles approaching 180°) when the cuticles are fouled by honey-dew.

In fact, it is generally impossible to obtain consistent contact angle values for species whose habitat is carrion, stored products, or similar organic media. Pal's observation on *Musca* is readily repeated, and similar great variations have been noted in *Dermestes vulpinus*. In *Tenebrio molitor* this phenomenon has been studied in more detail.

Ten pupae of this species were selected at random from a stock culture reared on damp bran and the contact angles of each prothorax measured. The following values were obtained:

Advancing	168,	154,	30,	168,	157,	112,	113,	80,	124,	103
Retreating	87,	68,	0,	64,	50,	45,	46,	0,	80,	53

All these specimens were mature, so that changes in angle with age (as will be described in a later section) were not responsible for this variation. The animals were examined alive, being immobile when attached to the supporting glass rods, and a group of five was kept in an incubator for 3 days, the angles being measured daily. It was found that the great variation persisted, although the angles showed some tendency to rise during the experiment, perhaps due to the washing off of loosely bound material (Table 3).

The wide variation, and low retreating angles, of *Tenebrio* pupae from culture are certainly due to the direct influence of the environment. Animals transferred to clean glass tubes during the last larval instar moult to pupae which show consistent surface properties.

A similar process of contamination has also been observed in *Rhodnius prolixus*, where it seems to be due to the fouling of the cuticle surface by excrement. After contamination the advancing and retreating angles may be as low as 30 and 0° respectively, instead of the normal values of 165 and 110°. The very great surface roughness of this species probably magnifies the effect of contamination. It is generally impossible to clean the surfaces by washing with water or other solvents once they have become badly fouled.

These examples illustrate very clearly the danger of using animals from unknown and uncontrolled environments for contact angle measurements. They also illustrate the typical, and diagnostic, effect of contamination, which is the production of a very wide range of variation between apparently similar specimens.

Table 3. *Persistence of contamination in* Tenebrio *pupae*

Specimen	Angle	Day			
		1	2	3	4
1	Advancing	30	75	80	70
	Retreating	0	0	0	0
2	Advancing	87	119	125	154
	Retreating	10	15	17	0
3	Advancing	124	126	137	140
	Retreating	80	78	86	69
4	Advancing	148	155	153	156
	Retreating	110	81	98	75
5	Advancing	103	97	Moulted	—
	Retreating	52	53	—	—

THE EFFECT OF ROUGHNESS ON THE CONTACT ANGLE

The effect of roughness on insect contact angles has been discussed briefly by Hoskins (1940) and by Pal (1951), and analysed in detail for an insect plastron by Crisp & Thorpe (1948). Several more detailed analyses of its effect have been made in the present study.

(*a*) *In* Tenebrio molitor *pupae*

When first formed the *Tenebrio* pupa is white, and its surface glossy in appearance. A wax layer covers the outside of the cuticle, but soon after pupation becomes covered by a cement layer. (The details of the structure and formation of these layers will be described elsewhere.) Darkening occurs at 10 hr. (at 25° C.), and at 12–14 hr. the secretion of a second, surface, wax layer begins. This process is complete by 30 hr., when the pupal surface is no longer glossy, but dull in appearance.

Examination of the pupal cuticle by reflection electron microscopy (Holdgate, Menter & Seal, 1955) has shown that the surface of the newly formed pupa is fairly smooth, apart from shallow ridging. The mature pupa, on the other hand, is covered with the closely packed fine filaments of the surface wax layer, which are about $0{\cdot}5\,\mu$ high, $0{\cdot}1\,\mu$ in diameter, and number 10^6–10^7 per mm.^{2}. These filaments are removed by solvents, and fused into a smooth film by heating to 80° C., and after both of these treatments the pupal surface is shown to be smooth by the electron microscope, and appears glossy to the eye.

The secretion of the surface wax layer is thus accompanied by great changes in the surface roughness of the pupa. In fact, it was the large accompanying changes in the contact angles which led to the discovery of this layer.

Experimental procedure

The animals were all removed from culture as last instar larvae and transferred to clean specimen tubes kept in an incubator at 25° C. The newly formed pupae were attached to clean glass rods by 42° C., melting-point paraffin wax, the attachment being by the ventral abdominal surface. They survived this process, continued to develop, and moulted to adult after the normal period.

The contact angles were measured on the prothorax and the specimens were returned to the incubator between measurements. A number of animals were examined at regular intervals of 2 or 3 hr. throughout the first day of pupal life; others were measured daily while yet other control specimens were examined at one known age only.

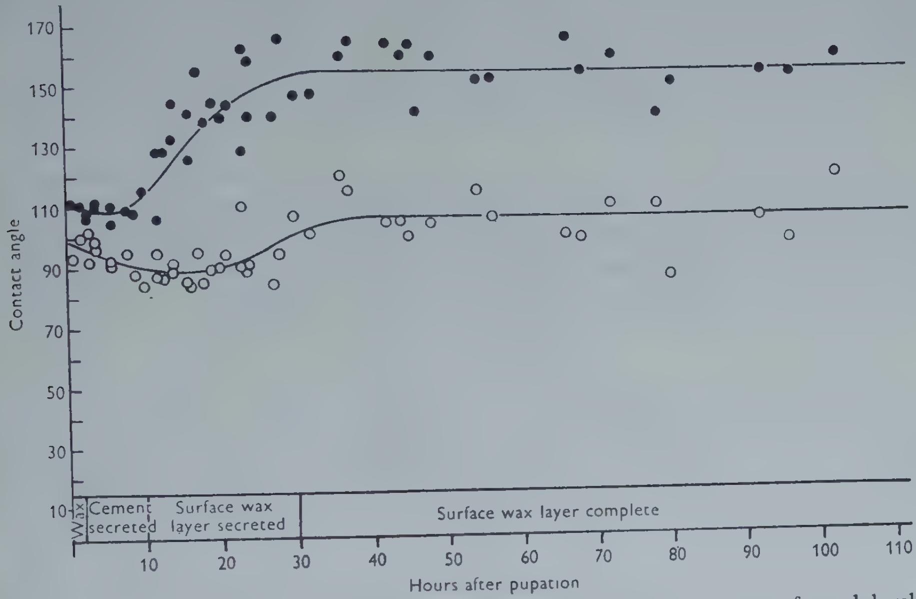

Fig. 1. Changes in contact angle in *Tenebrio molitor* pupae during the early stages of pupal development. Solid circles: advancing angles; open circles: retreating angles. The changes in the nature of the epicuticle surface are indicated along the base of the figure.

Results

The changes in the contact angles of the pupal prothorax during the first 30 hr. of development (at 25° C.) are shown in Fig. 1. It is evident that the very large increase in the advancing angle, from about 110° in the young pupa to about 155° in the mature specimen, is closely correlated with the secretion of the surface wax layer. Further evidence for this correlation was obtained from specimens kept at 20° C., at which temperature the secretion of the surface wax layer is delayed and the rise in contact angle occurs later and more gradually.

The action of solvents and heat in rendering the surface smooth have already been described. These agents also produce a very great fall in the advancing contact angle, which returns to a value similar to that of the young pupa (Table 4).

The changes in contact angle are thus directly related to changes in roughness. They cannot be due to changes in the chemical composition of the cuticle surface since the advancing angle of even the young smooth-surfaced pupa is near to that of paraffin wax, and not far below 120° which is generally considered to be the theoretical maximum for a smooth surface (Adam, 1948). The much larger angle of the mature animal must be due to increased roughness.

Table 4. *The effect of various treatments on the contact angles of mature* Tenebrio *pupae*

Normal values		After heating to 80° C.		After chloroform for 1 min.	
Advancing	Retreating	Advancing	Retreating	Advancing	Retreating
140	93	106	75	107	86
163	110	110	83	111	89
155	100	105	85	111	93
160	104	113	97	110	95

If it is assumed that the *true* contact angles of the pupal surface undergo no change during the period under consideration, and are close to the value for paraffin wax, it is possible to calculate the roughness of the surface, and the extent of trapped air below the interface. According to Cassie & Baxter (1944):

$$\cos \theta'_a = f_1 \cos \theta_a - f_2$$

and

$$\cos \theta'_r = f_1 \cos \theta_r - f_2,$$

where θ'_a is the apparent and θ_a the true advancing angle, θ'_r and θ_r the apparent and true retreating angles, f_1 the area of liquid-solid interface per unit of projected surface area, and f_2 is the area of liquid-air interface per unit of surface area.

For *Tenebrio* pupae, $\theta'_a = 155°$, $\theta'_r = 110°$, by observation, and $\theta_a = 105°$, $\theta_r = 92°$, by assumption. Using these values it is now possible to solve the equations for f_1 and f_2. The results obtained are $f_1 = 2{\cdot}52$; $f_2 = 0{\cdot}25$.

The surface of the mature pupa thus retains trapped air over a quarter of its projected area, while the remaining three-quarters make contact with the liquid. But, owing to the great roughness of the surface, these three-quarters of the projected area have a true area of 2·52 times the *total* projected area. If the areas covered by the air film had the same surface contour, this would suggest a 'roughness factor' (i.e. ratio of true area to projected area) of 3·36 for the cuticle as a whole. This treatment is not, however, really valid, for the air may be retained in deep pits whose true area could be very great. As an approximation, it is probably justifiable to state that the ratio of real area to projected area in the mature *Tenebrio* pupa is about 3·5. Inspection of the reflexion electron micrographs shows that the roughness of the surface wax layer is certainly of this order, and is therefore adequate to produce the large observed angles without there being any difference in chemical composition between it and the surface layer of the young pupa.

The pupa of *Tenebrio* is an especially valuable example because it allows a change in contact angle to be correlated with a change in surface roughness and with a process of cuticle secretion. Equally clear evidence of the effect of surface irregularities can be found in many other species, and it is probable that all the insects listed in group 1 of Table 2 owe their large apparent angles to this factor alone. Their true angles need not differ significantly from those of the smooth-surfaced terrestrial species which form group 2 of the table.

(*b*) *In* Calliphora erythrocephala

The highest contact angles observed in insects are found on surfaces covered with a hair pile, and in these cases both advancing and retreating angles may approach 180°. None the less, the contact angles of the individual hairs, where these have been measured, are not nearly so large. Pal (1951) states that the hairs on the dorsal surface of *Arctia caja* larvae have an advancing angle of 97°, although the surface as a whole has an apparent angle of 180°. Crisp & Thorpe (1948) consider that complete waterproofing of a plastron can be obtained with angles of even less than 90°, while angles of just above 90° would make a hair pile extremely resistant to water penetration.

In the present work some observations have been made on the abdominal surface of the adult *Calliphora erythrocephala*, which is covered with large hairs and is normally unwetted by water. The apparent advancing angle is about 175°, and the apparent retreating angle 155°. Individual hairs, however, show advancing angles of 110°, and retreating ones of 95°, these measurements being very approximate owing to the small size of the structures involved.

Applying Cassie & Baxter's equation, treating the angles for the hairs as the true ones for the surface, f_1 is found to be 0·28 and f_2, 0·90. Nine-tenths of the surface are thus covered with trapped air (a value which observation of the surface suggests to be quite reasonable), and for the remaining tenth, the 'roughness' of the hair tips causes the true area to exceed the projected by a factor of 2·8. The extremely high contact angles of the surface thus result from the structure and spacing of the hairs and not from any abnormal water-repelling properties of their surface molecules. In this they resemble other highly efficient water-repelling biological surfaces such as duck feathers (Cassie & Baxter, 1945).

(*c*) *In* Anax imperator *nymphs*

If the true contact angles of a surface are below 90°, and the surface is rough without being deeply porous, the roughness acts to lower the observed angles (Wenzel, 1936). An example of this phenomenon is to be seen in the nymphs of Odonata, and in other rough-surfaced aquatic species (group 4, Table 2). It has been studied in detail in the nymph of *A. imperator*.

In this animal, the general body surface is covered with short appressed spines, which do not overlap, but render the surface very rough. The apparent advancing contact angle on these regions (usually measured on the dorsal abdominal surface) is about 45°, and the retreating angle is 0°. The smoothest part of the surface, the cuticle over the compound eyes, has an advancing angle of 71° and a retreating angle of 5–10°.

If the angles measured on the eyes are regarded as near the true values for the smooth cuticle, the roughness factor of the abdomen can be calculated by the equation of Wenzel (1936)

$$\cos \theta_d = r \cos \theta_t,$$

where θ_d and θ_t are the apparent and true angles, and r is the roughness factor.

Substituting for the advancing angles in this expression, the roughness factor is given as 2·2. This is in fair agreement with subjective estimates, but it is, of course, desirable to check it by another method.

If the surface could be coated with a thin layer of a substance of known surface properties, and the apparent angles then measured, the roughness factor could be calculated directly. This method was applied to the *Anax* nymph, using thin films of paraffin wax laid down from chloroform solutions. After treatment, the mean advancing angle for four insects was 123°, and the retreating angle 86°, the variation between individuals being small. Little or no air was trapped on the surface, so that Wenzel's equation could be applied directly. The true advancing angle of paraffin wax is the subject of controversy, and it is in any case doubtful whether the film will crystallize smoothly; on the other hand, it is likely to obliterate small surface irregularities. If the true advancing angle is taken as 100°, then r is given as 3·1; if 105° is assumed, then $r = 2·1$. Of these the latter is probably the more nearly correct. It is now possible to find the true angle for the normal nymph, given the apparent angle of 45°, and r. If r is taken as 3·1, θ_t is 77°; if $r = 2·1$, $\theta_t = 70°$. These values are in good agreement with the direct measurements on the compound eyes, and indicate that very low apparent angles may be produced by surface roughness. For retreating angles, it is found that for an apparent angle of 0° to be produced the true angle must not exceed 60°.

It is therefore evident that the very low contact angles of the *Anax* nymph could be obtained, with the observed surface roughness, if the true advancing angle was about 70–75°, and the retreating angle was about 60°, or less. This immediately raises the question of whether these values could be obtained from a cuticle whose chemical composition was the same as that of terrestrial species, if the prolonged contact with water to which the aquatic larva is exposed produced a lowering in angle similar to that known from other surfaces.

The three examples considered in detail indicate very clearly the effect of surface texture on the contact angle. It is evident that the great scatter of values shown in Table 2—advancing angles ranging from 35° to 175–180°—is enormously reduced when allowance is made for this factor. It is probable that no insect has a true advancing angle of more than 110°, or less than 65°, and it is now necessary to consider the factors that may be responsible for the production of this reduced range of variation.

THE EFFECT OF WATER ON THE CONTACT ANGLE

Despite the emphasis, so prominent in the physical literature, on the importance of contact with liquid as a factor modifying the contact angle, this factor has never been considered in the biological work on wetting properties. A number of experiments have therefore been undertaken in order to determine the effect of prolonged soaking in water on the contact angles of insects.

Experimental procedure

In all these experiments the method was essentially the same. The specimens—intact living insects, dead insects or detached wings and elytra—were attached to glass rods by wax or cellulose paint and immersed in tap water. In some cases a steady flow was kept up throughout the period of immersion; more usually the animals were submerged in dipping jars filled with water that had been boiled to remove dissolved air and prevent the formation of bubbles. The angles were measured at regular intervals and the water changed daily.

Results

(*a*) *In living* Tenebrio *pupae*

Fig. 2 shows the effect of this treatment on living *Tenebrio* pupae, which were supported with the abdomen out of the water so that respiration was not impaired. The angles are for the prothorax. It is evident that there has been a rapid and extensive lowering in both angles, the retreating angle in particular falling almost to 0°. The surfaces, however, still retain an air film among the wax filaments, and this has probably protected the deeper hollows from the water.

The apparent advancing angle after soaking for 24 hr. is about 115°, and the apparent retreating angle, 10°. It is probable that the values of f_1 and f_2 in Cassie & Baxter's equation will not have changed greatly, especially since f_2, which is the most liable to alteration, is small. We can therefore calculate the true angles for the soaked pupa, and these are given as 94° (advancing) and 61° (retreating). There has thus been a considerable fall in the advancing angle, but a much greater fall in the retreating one.

(*b*) *In other species*

Similar curves for the lowering in angle with time are plotted for five other species in Fig. 3. In all these insects the results are similar to those in *Tenebrio*, the retreating angles being much more affected than the advancing ones. It is noteworthy that in four of these species the advancing angle changes from above 90° to below 90° during the experiment; this fact serves to eliminate changes in roughness (and contamination of the surface of the test liquid were this not otherwise excluded) as the cause of the observed change.

There can be no doubt, therefore, that prolonged contact with water produces a very great alteration in insect wetting properties. It also appears likely that soaking for long periods would produce a fall in angle to values similar to the true ones observed in *Anax* larvae, and similar readily wetted aquatic species. The low angles of these animals could therefore be a direct effect of their environment, and the composition of the cuticle need not differ from that of terrestrial species. This conclusion is in agreement with the observation of Crisp & Thorpe (1948) that in the bug *Aphelocheirus* the larva is very hydrophobic when newly moulted, but after immersion for some months the contact angle of the outer surface falls to 10–20°.

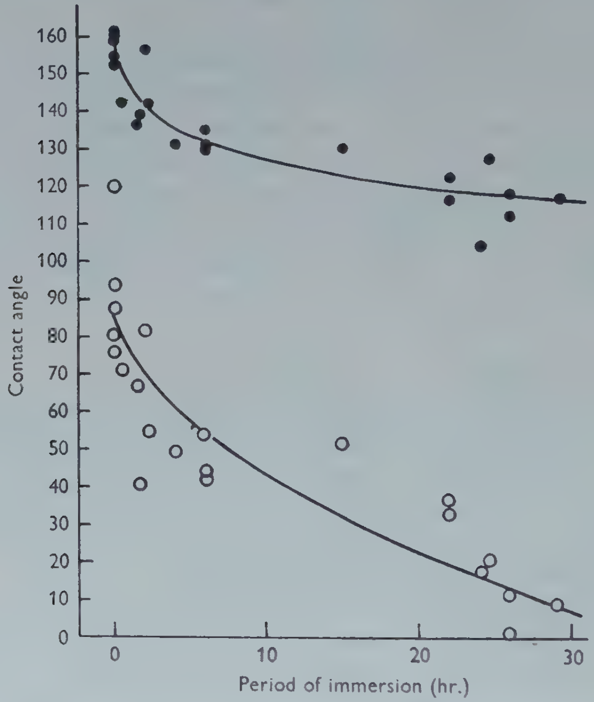

Fig. 2. Changes in the contact angle of *Tenebrio molitor* pupae following immersion in water. Solid circles: advancing angles; open circles: retreating angles.

It is not surprising to find aquatic insects with low contact angles, but it is remarkable that a number of species that live wholly or partially submerged maintain large and constant angles. Some of these appear in group 3, Table 2, and with them may be included *Gyrinus* (group 2), which, though surface living, maintains large angles on its submerged ventral surface. To some extent the smooth surfaces of the insects of this type may be responsible for the large angles; there is no tendency for them to be lowered below the true values. But, as will be seen from Fig. 3, these large angles which appear constant for freshly killed material are just as much affected by soaking in water when the animals are dead as are those of terrestrial species. The figure shows curves for *G. marinus* and *Naucoris cimecoides*, and both of them clearly conform to the normal pattern.

From these observations it appears probable that certain aquatic insects actively maintain high and constant contact angles during life. It would appear significant that these species are either surface living, surface breathing, or in the habit of occasionally leaving the water. No satisfactory mechanism can be established at present for this maintenance of a large contact angle, but it is interesting to recall that various 'oil-secreting' glands have been described as opening on the general surface, or around the spiracles of many aquatic insects (Richards, 1951; Keilin, Tate & Vincent, 1935). For these secretions to be effective, however, they must have few hydrophilic polar groupings, and have little tendency to spread in a water surface.

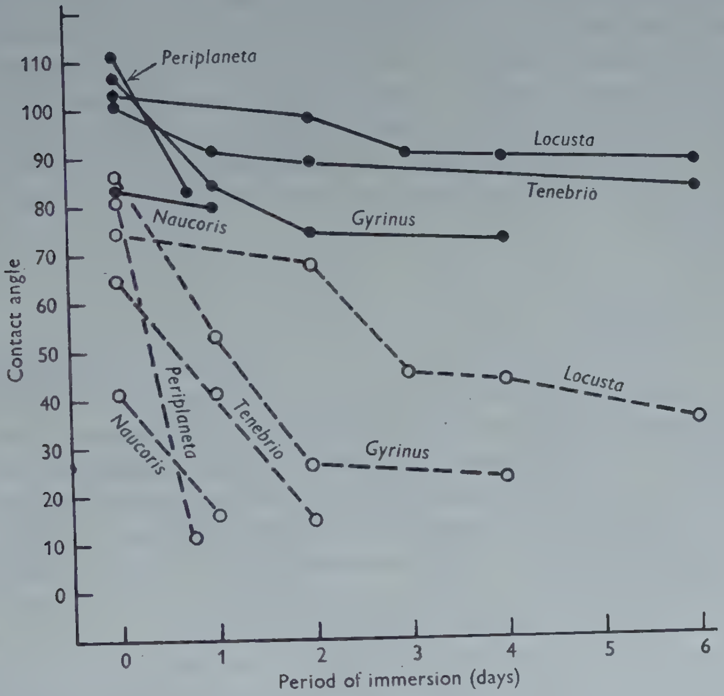

Fig. 3. Changes in the contact angles of several species following immersion in water. Solid circles and continuous lines: advancing angles; open circles and broken lines: retreating angles.

A similar problem of maintaining a large contact angle occurs in many, if not all, insects in the tracheal system. Crisp & Thorpe (1948) have pointed out that in *Aphelocheirus* the moulting fluid will not be expelled from the plastron unless the retreating contact angle is brought up to 90°, and this will have to be done by the insect against the attraction of the fluid in contact with the surface. Similarly, in a tracheal system, Wigglesworth (1953) has pointed out that air will readily emerge from a saturated solution in contact with a hydrofuge surface such as is believed to line the tracheae, but for this system to be possible the material of the lining will have to maintain a high retreating contact angle against the fluid it contains.

Before any useful suggestions can be made about the possible mechanisms involved in this process it is necessary to decide as to the cause of the lowering in angle produced by soaking. There has been some dispute in the past on this subject; removal of air or contaminant films, 'penetration' of liquid into the solid, and re-orientation of polar molecules have all been postulated. At present, the experimental evidence seems to favour the last of these hypotheses (Rideal & Tadayon, 1954). There are certainly large amounts of polar material present in the insect epicuticular waxes, which consist of mixtures of long-chain alcohols, paraffins, acids and esters (Chibnall, Piper, Pollard, Williams & Sahai, 1934). In many species, too, these waxes are exposed on the cuticle surface (*Tenebrio* pupa (Holdgate, unpublished work); *Calliphora erythrocephala* (Wolfe, 1954); *Blattid* spp. (Ramsay, 1935; Dennell & Malek, 1955; Beament, 1955); *Rhodnius prolixus* (?) (Wigglesworth, 1945); possibly various Hymenoptera (Glynne Jones, 1955); possibly *Chilo simplex* (Miyazaki & Koidsumi, 1952), and even where a cement layer is present this may well incorporate lipids (Beament, 1955). Re-orientation of these molecules at the interface would therefore provide a possible explanation for the lowering produced by soaking, and this possibility was further investigated in some detailed studies on the cockroach, *Periplaneta americana*, and the locusts *Locusta migratoria* and *Austroicetes* sp.

(*c*) *Contact angle and humidity relations in the cockroach*

The cuticle surface in the cockroach is covered with a fluid grease composed of long-chain paraffins and alcohols solubilized by the incorporation of a large proportion of short-chain members of the same series (Beament, 1955). When immersed in a surface film this grease spreads from the cuticle and stable contact angles are not observed, but ultimately spreading ceases and consistent angles may be measured. These angles are probably governed by a residual monolayer of grease, which is firmly bound to the surface. Cuticles thus briefly washed show a normal lowering of the contact angles after prolonged immersion in water, and a curve for this is shown in Fig. 3.

In this fresh material, the retreating angles do not fall to 0° unless soaking is continued for more than 24 hr. Much more rapid changes are observable in thoroughly dried cuticles, from which the short-chain 'solvents' (Beament, 1955) have evaporated, leaving a residual coating of hard wax. Some very old exuviae (kindly provided by Dr J. W. L. Beament) had an advancing angle of about 110°, and a retreating angle of about 45° when first examined, but the retreating angle showed signs of change even during the process of measurement and fell to 0° within 30 min. During this period the advancing angle fell to about 75°. These dry, solvent free, cuticles thus differ from the fresh material in two respects; their retreating angles are in any case low, and these fall to 0° very soon after immersion in water.

It is noteworthy that the surface properties of these dried exuviae closely resemble those of beeswax or the hard wax fraction of cockroach grease, when these materials are allowed to solidify from a melt in air or are deposited from a chloroform solution. Smooth beeswax surfaces, for example, have an advancing angle of 88° and a retreating angle of about 30° when first examined, but the retreating angle falls to 0° after only about 1 min. in water. It is likely that these low retreating angles are due to the polar nature of the molecules (Chibnall *et al.* 1934), and that in these solid waxes the molecules are not firmly bound and so can undergo re-orientation at the interface.

Exposure to air for long periods also produces marked changes in the contact angles of cuticles from which the bulk of the grease has been removed by washing in a water surface. As in the dried exuviae the retreating angles are particularly affected (Table 5). During the period of exposure the cuticles (which are, of course, shielded from atmospheric contamination) dry out and become brittle, and also lose 'solvent' by evaporation. The remaining grease film is thus likely to become disorientated during this period, and the lowering in angle may be due to this cause.

Table 5. *The effect of 14 days' exposure to air on the contact angles of the cockroach*

Specimen	Initial values		After exposure	
	Advancing	Retreating	Advancing	Retreating
1	106	95	92	70
2	110	94	91	58
3	105	90	71	50
4	105	94	77	44
5	104	92	60	53

The low contact angles of cockroach cuticles produced by immersion in water persist after the cessation of the treatment. Even exposure to air for a month, or desiccation for a week, fails to alter the angles. This observation seems to eliminate two out of the three possible mechanisms for the lowering in angles by soaking. If the fall in angle were due to the replacement of an adsorbed air film by one of water, or to the 'penetration' of the water into the cuticle to an appreciable depth, it seems probable that this adsorbed or incorporated water would be removed by desiccation. At least, this treatment ought to produce a partial restoration of the initial large angles. But if the water acts by increasing the number of polar groups in the exposed surface, there is no reason why desiccation should cause any re-orientation, since there is no water phase either inside or outside the cuticle.

Further evidence that the lowering in angle depends on re-orientation of polar molecules is provided by the effect of saturated air. If fresh cockroach wings are placed in a closed chamber containing a water surface, but are not allowed to come into contact with it, their contact angles undergo no change. But if wings whose angles have previously been lowered by soaking in water are placed in this humid air, there is a considerable rise in both the advancing and retreating values. Fig. 4 contains the results from an experiment of this kind. This experiment, again, eliminates adsorption of water as a possible mechanism for the lowering in angle following soaking, since it is hardly likely that such an adsorbed film, which cannot be removed by desiccation, would yet be replaced by air in an atmosphere saturated with water vapour. It seems probable that the rise in angle in damp air is due to the absorption of water by the cuticle, and the restoration of a water phase inside the wing—which becomes much more flexible during the process. The rise in angle in this experiment, like the lowering during immersion, is thus interpreted as being due to a re-orientation of polar molecules by the attraction of the liquid water.

There are striking parallels between these observations and those of Beament (1955) on the properties of the cuticular grease of the cockroach. In particular, the contact angle observations suggest that the presence of the short chain 'solvents' are essential for the maintenance of stable surface properties, and that the presence of a water phase inside the cuticle is also important. It is noteworthy that Beament has similarly shown that orientated films of cockroach grease cannot be formed except on an aqueous surface.

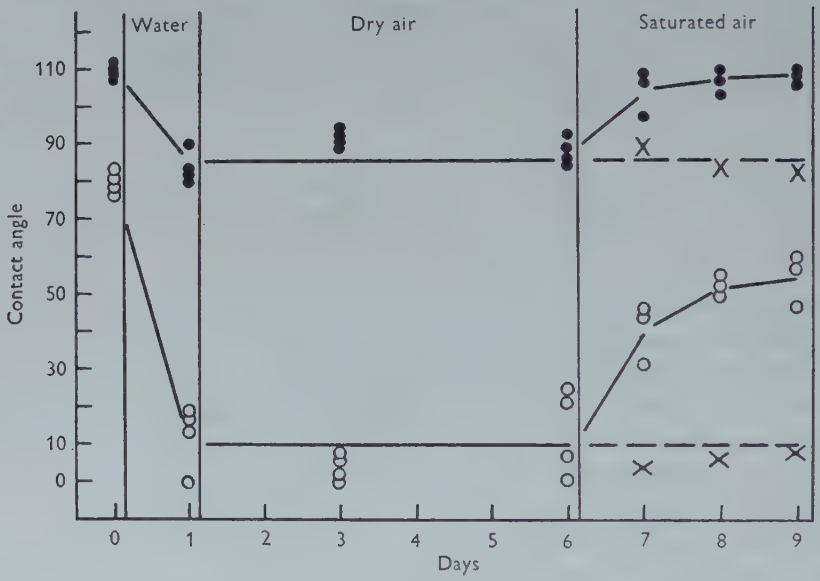

Fig. 4. The effect of various treatments on the contact angles of a group of four *Periplaneta* forewings. Solid circles: advancing angles; open circles: retreating angles. The crosses show the advancing and retreating angles of a control specimen which was left in the desiccator when the others were transferred to humid air.

(*d*) *Contact angle and humidity relations in locust cuticles*

A series of experiments parallel to those described above has been carried out on the wing cuticles of the locust species *Locusta migratoria* and *Austroicetes* sp., these wings being especially suitable material for study by the 'inclined plate' method.

The results obtained are similar in all respects to those from the cockroach. Soaking in water produces a marked lowering in contact angle (see Fig. 4), although this proceeds rather more slowly than in *Periplaneta*, and drying also causes a lowering. Further, the angles of thoroughly dried *Austroicetes* wings rise steadily when the specimens are placed in saturated air, and finally attain values close to those of fresh material. It would seem likely, therefore, that in these insects also the surface properties depend on a layer of polar molecules.

Summarizing these experiments, there is no doubt that the contact angles of insects are profoundly affected by the water relations of the surfaces prior to measurement. Where detailed studies have been carried out, it would appear that the results are most readily explained in terms of the orientation of polar molecules.

CONTACT ANGLES AND THE CUTICLE

(*a*) *Contact angles and the moulting cycle*

The existence of changes in wetting properties that can be correlated with processes of cuticle secretion was first demonstrated by Wigglesworth (1947) in *Rhodnius*, in the immediate post-moulting period. Some measurements of the contact angles during this period were made by Pal (1951), but his results did not indicate any marked changes. In the present work some further measurements of the contact angles of the newly moulted *Rhodnius* have been made; these are given in Fig. 5 and are in obvious agreement with Wigglesworth's original description.

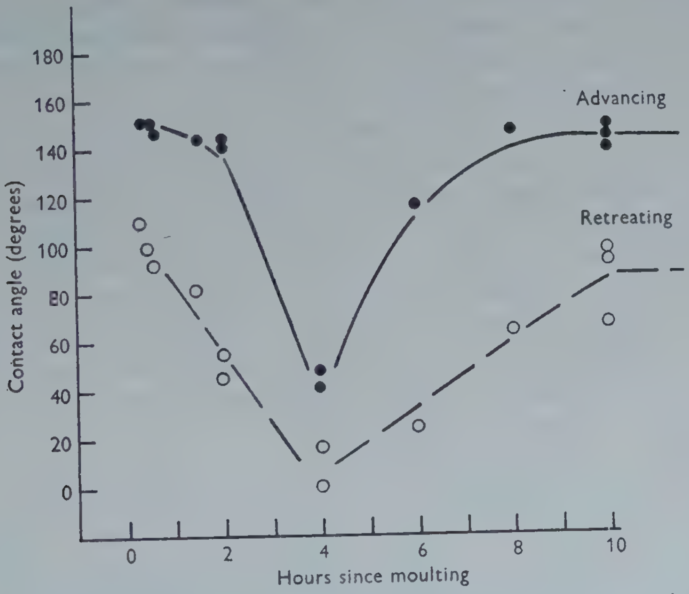

Fig. 5. Changes in contact angle in *Rhodnius prolixus* 5th-instar nymph following moulting.

These variations in contact angle are undoubtedly exaggerated by the great roughness of the *Rhodnius* cuticle. In the newly emerged insect when a wax layer is exposed on the surface, and in the later stages when cuticle secretion is complete, the apparent contact angles are about 160° (advancing) and 110° (retreating). But observation shows that in these stages trapped air makes up a large part of the interface, and the values for f_1 and f_2 (obtained by coating the surface with paraffin wax, which gives apparent angles almost identical with those of the untreated animal) are: $f_1 = 2{\cdot}2$, $f_2 = 0{\cdot}58$. The true angles for the surface in both stages are therefore close to those of paraffin wax, and are given as about 100° (advancing) and 80° (retreating).

In the 'spreading' stage, during cement secretion, when the apparent advancing angle is only 50°, and the retreating angle about 10°, air does not make up an appreciable part of the interface. The apparent angles may therefore be related to the true values by Wenzel's equation, the roughness factor being taken as 4. The true advancing angle is found to be about 80°, and the retreating angle 75°.

Correction for roughness, therefore, reduces the variation in angle in the moulting *Rhodnius* in the same way as it reduces inter-specific variation. The residual values are close to those found by Pal (1951) using a droplet method, which involves much smaller areas of surface and may not be so affected by roughness. This correction removes the apparent divergence between the wetting properties of the newly moulted *Rhodnius* and those of *Tenebrio* during the same stage. Wigglesworth (1948) noted that in the adult *Tenebrio* cement secretion was only accompanied by minor changes in wetting properties, and reference to Fig. 1 will show that in the pupa of the same species cement secretion involves a fall in angle of only 5–10°. But both adult and pupa of *Tenebrio* have nearly smooth surfaces at this stage, so that there is no tendency for these variations to be exaggerated. In contrast, the secretion of the surface wax layer in *Tenebrio* pupae, with the consequent roughness changes, produces an enormous increase in contact angle.

Another great change in the contact angles during moulting occurs in the Odonata, when the last stage nymph moults to adult. In *Coenagrion*, for example, the nymphal advancing angle is about 35–40°, and the retreating angle 0°, while the adult angles are both near to 180°. But in both cases the surfaces are very rough, and it is probable that the true advancing angle changes only from about 70° to about 105°. Much of this change can probably be accounted for by the change in habitat.

It is clear that the principal changes in contact angle during the moulting cycle depend on changes of surface contour rather than composition. Whenever two instars differ in the roughness of their surfaces, they will almost certainly differ in wetting properties. Whenever a change from aquatic to terrestrial habitat occurs, similar changes in contact angle are likely.

In the adult *Tenebrio molitor* there is a change in contact angle during the first few days after emergence that is not attributable to roughness changes (Fig. 6). This consists of a slow fall in the retreating angle (measured on the elytra). Similar results have been obtained from insects kept in clean glass tubes and examined at one known age only, and from others which were attached alive to glass rods by paraffin wax and measured daily. Experimental method is thus eliminated as the cause of the observed change and it is likely that the animal itself is responsible for the change, which probably results from the secretion of some loosely attached polar material on to the surface. Washing in xylol for 1 min. removes this material, and causes the angles to return to values like those of the newly moulted animal.

(*b*) *Contact angles and cuticle composition*

It has already been shown that the wide variation in insect wetting properties does not provide any evidence for similar variations in cuticle composition. Almost all the contact angle phenomena observed can be accounted for if the surfaces involved have true advancing angles of about 100–105° and retreating angles of 85–95°, and if their behaviour in relation to roughness and water contact is normal. It has been shown experimentally that this latter condition is fulfilled; it now remains to consider the implications of the former requirement.

A true advancing contact angle of 100–105° is most characteristic of surfaces consisting largely of $-CH_3$ groupings, and such surfaces will also have retreating angles of about 90°. A great deal of the insect cuticle probably consists of material capable of forming such surfaces. Insect waxes can do so, if the polar groups which they contain are directed away from the surface and bound so that they do not respond readily to the re-orientating force of the water. Thus it is that beeswax, *Tenebrio* wax, and hard cockroach wax (from a sample kindly provided by Dr J. W. L. Beament) all show low retreating angles when deposited from chloroform solution or allowed to solidify in air; for them to be responsible for the observed contact angles of insects it appears necessary for such waxes to be regularly orientated and firmly bound. Beeswax deposited on to grease free cockroach wings or on to shellac surfaces from octane-octyl alcohol solutions appears to be orientated in this way initially, and has an advancing angle of 110°, and a retreating one of about 85°. But the retreating angle begins to fall immediately after immersion, and reaches 0° in about 15 min. It would appear, therefore, that some more rigid binding mechanism must exist if such materials are to form surfaces with constant, large advancing and retreating angles.

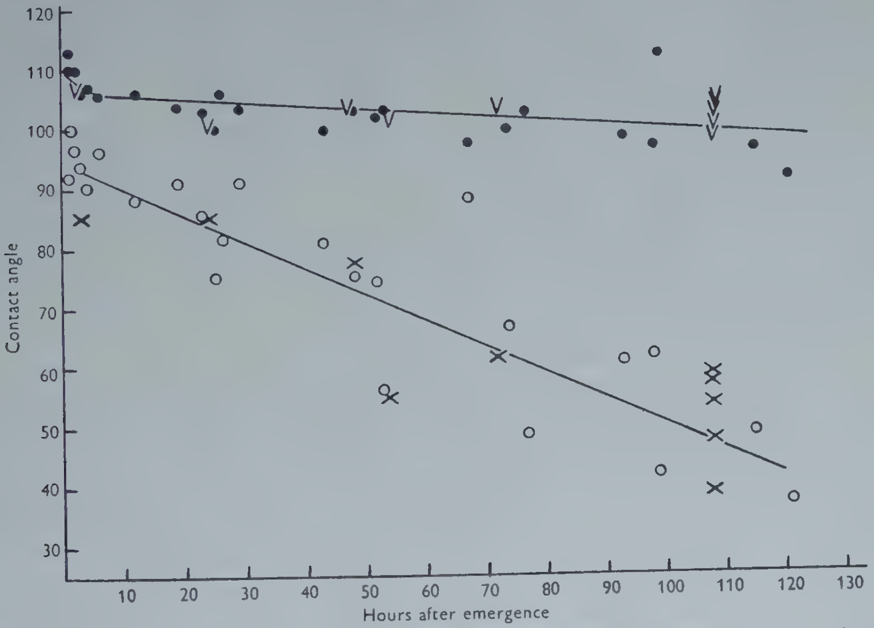

Fig. 6. Changes in contact angle in adult *Tenebrio molitor* with age. The solid circles are advancing angles for animals which remained attached to the mounting rods throughout the experiment; the *V*'s are advancing angles for individuals examined at one known age. Open circles and crosses are similar figures for retreating angles.

But the chloroform-soluble insect waxes are not the only epicuticular components capable of producing large contact angles. Wax solvents such as chloroform have very little effect on insect contact angles, even when the animals are immersed in the boiling reagent for long periods (Table 6). This observation is not, however, really surprising. Although extraction with boiling chloroform leads to the complete removal of the cuticular waxes (Beament, 1945), it has no effect on the resistant lipoprotein or cuticulin layer below them. This layer has not only been tanned, a process which generally renders protein layers hydrophobic (Pryor, 1940), but also contains bound long-chain paraffin molecules (Dennell & Malek, 1955). It would therefore be expected to have few exposed polar groups and to differ little in its wetting properties from paraffin wax.

Table 6. *The effect of wax solvents on insect contact angles*

Species		Normal angles	Angles after cold wax solvents			Angles after hot wax solvents	
			Number	Chloroform	Xylol	Number	Chloroform
Periplaneta americana	Adv.	106	4	110	—	4	108
	Ret.	91		66	—		60
Austroicetes sp.	Adv.	107	4	100	—	4	103
	Ret.	79		68	—		73
Locusta migratoria	Adv.	107	2	—	110	2	105
	Ret.	93		—	91		77
Tenebrio molitor	Adv.	107	5	—	107	7	104
(adults)	Ret.	92		—	92		82
Anax imperator	Adv.	45	—	—	—	3	94
(nymphs)	Ret.	0		—	—		0

Adv. = advancing; ret. = retreating.

Indeed, the similarity between the contact angles of the wax-free cuticle and those of the waxes themselves is so great that the removal of a surface wax layer may easily go undetected unless it is accompanied (as it is in *Tenebrio* pupae) by changes in roughness. It is not justified, therefore, to assume that because the hydrophobe properties of a cuticle persist after treatment with a wax solvent or detergent they were not originally due to wax.

Besides resembling the unextracted cuticles in their general properties, the lipid-free surfaces are liable to show a typical lowering in contact angle on immersion in water. This might be held to indicate that the extraction is not fully efficient; but even if polar molecules capable of re-orientation are absent, a lowering in angle is still possible by adsorption or by penetration of water into the freely permeable cuticle.

It is clear, therefore, that the observed properties of the insect surface could be due to a number of different cuticular components, and that contact angle measurements are not likely to provide a useful method for the detection of inter-specific variations in surface composition.

DISCUSSION: BIOLOGICAL ASPECTS

A species living in a highly organic medium, and liable to extensive contamination, has very variable surface properties which do not appear of any importance to it. Other terrestrial species, on the other hand, usually have large and fairly constant contact angles, which are probably of survival value since they render the animals resistant to wetting by rain and other water surfaces they may encounter.

As might be expected, the clearest evidence of genuine adaptation of wetting properties to a particular mode of life is seen in surface-living aquatic species. Hair piles are developed in the pond skaters, and in *Gyrinus* the smooth cuticle is structurally modified so that it produces a constant water-level on the body no matter whether the animal enters the surface film from above or below. In submerged species adaptations in the form of hair piles are well known (Thorpe, 1950). Other permanently submerged stages with closed tracheal systems or long breathing tubes which can be extended to the surface often have readily wetted cuticles which probably owe their properties to the direct influence of the environment.

In the majority of insects such adaptations as occur are structural rather than chemical. It appears 'easier' for a species to become adapted to an environment by changing the contour rather than the composition of its surface. There is good reason for this. The majority of adaptations of insect surface properties involve increases in contact angle, achieved by the formation of hair piles and the consequent retention of air films. To do this changes in roughness are obligatory, since the insect already has contact angles which are near the upper limit for smooth surfaces.

In certain aquatic species the contact angles appear to be actively maintained at constant large values during life. In these the problem is to maintain a retreating angle of about 90° despite the complete immersion of the animal in water. A similar problem is present in the tracheal system or plastron when it is newly secreted; if it is to fill with air according to the mechanism proposed by Wigglesworth (1953) it is necessary for the retreating angle to be large, at least for a short time after secretion. Further, if insects with a surface layer of polar wax are to maintain large advancing and retreating angles, it is necessary that the molecules of this wax should be secreted and maintained in an orientated layer. It seems likely that all these problems may be met in essentially the same way, perhaps first by secreting the wax as a packed film in a manner analogous to cockroach grease (Beament, 1955), and secondly by some mechanism of preferential attraction of polar groups away from the surface. It is possible that the cement layer, at least in some insects, consists of an intimate mixture of wax and other materials and it may be the binding in this layer which is responsible for the maintenance of the normal wetting properties of the surface.

SUMMARY

1. The water contact angles of insects show a wide range of variation, which is broadly correlated with surface roughness and with habitat.

2. The contact angles of species inhabiting stored products or carrion are greatly modified by contamination. This produces large variations between apparently similar individuals.

3. In terrestrial insects surface roughness increases the contact angles to very large apparent values. Detailed analyses of its effect have been made in the pupa of *Tenebrio molitor* and the adult *Calliphora erythrocephala*. In some aquatic insects surface roughness leads to a reduction in the contact angles; this has been studied in the nymph of *Anax imperator*.

4. Prolonged immersion in water causes a lowering of the contact angles of all the insects examined, and the low angles of many aquatic species may therefore be the direct effect of their environment. In some aquatic species there is evidence of the active maintenance of a large contact angle during life.

5. Changes in contact angle accompany processes of cuticle secretion and will occur at any moult if changes in roughness or habitat take place.

6. The observed variations of surface properties can be explained without assuming any variation in the chemical composition of the cuticle surface. Wetting properties are of little value as indicators of cuticle surface composition.

7. The biological aspects of insect surface properties are briefly discussed.

My thanks are especially due to Dr R. H. J. Brown for advice on the design and construction of apparatus, and to Prof. V. B. Wigglesworth and Dr J. W. L. Beament for advice and stimulating discussion at all stages of the work. I am also most grateful to Mr G. D. Glynne Jones and Dr J. W. L. Beament for allowing me to see the typescripts of papers prior to publication.

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APPENDIX. APPARATUS FOR CONTACT ANGLE MEASUREMENT

Fig. 7 shows the general appearance of the apparatus, viewed from the front. The specimen (*a*) dips into the water surface contained in the vessel (*b*), which is supported on the box (*c*). A large Perspex wheel (*e*) is mounted on the same axis as the specimen holder and has a protractor scale engraved on its face. A friction band passes around the rim of this wheel, and around a smaller wheel on the same shaft as the knob (*d*), so that the inclination of the specimen to the vertical may be altered by rotation of (*d*). This inclination may be measured using the protractor scale.

The platform on which the specimen holder and rotation system are mounted is slung from the rails (*h*) by four levers (*f*). A vertical movement is imparted to this platform by rotation of the knurled wheel (*k*), which, by means of the two screws (*l*, *m*) of different pitch, causes the two pairs of wheels (*g*, *n*,) to approach one another at equal rates.

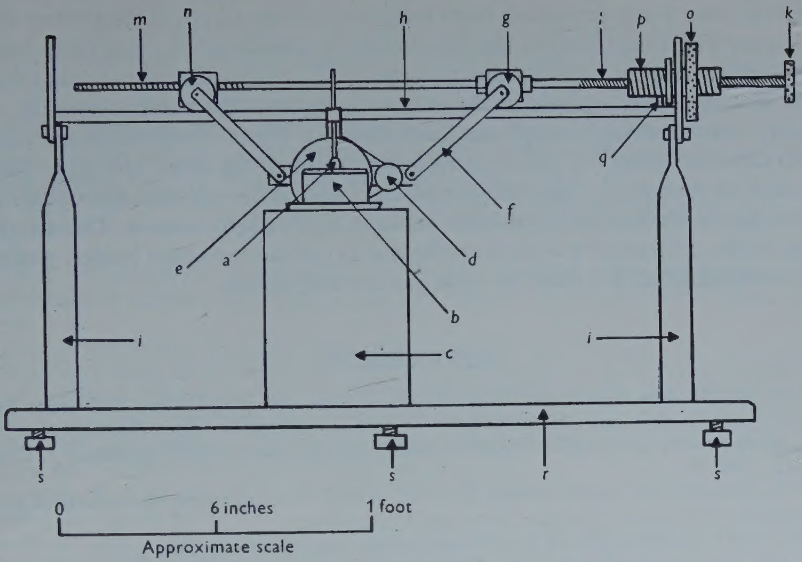

Fig. 7. The apparatus used for contact angle measurement, front elevation. For explanation see text (Appendix).

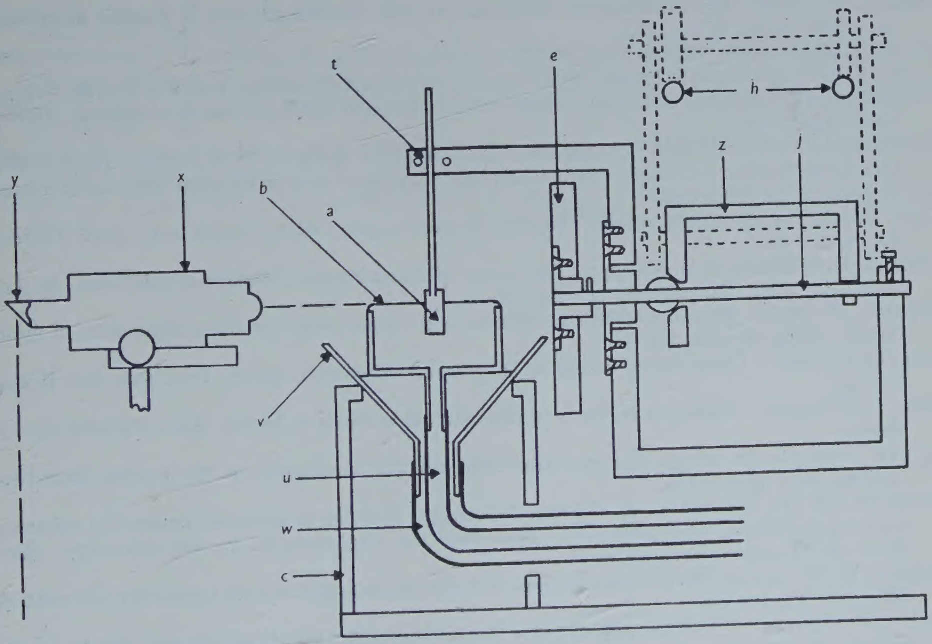

Fig. 8. Diagrammatic section through apparatus in the plane of observation and of the specimen mounting. For explanation see text (Appendix).

A horizontal movement is given by rotation of the wheel (*o*), which advances or withdraws the screw (*p*), this being prevented from rotation itself by a key (*q*) running in a keyway. The supports (*i, i*) are fixed to the baseboard (*r*), which stands upon three bolts (*s*).

The specimen mounting and the arrangement of the water surface are further illustrated in Fig. 8. The specimen (*a*), held in the clamp (*t*), dips into the water surface (*b*), which is renewed by water entering through the inner tube (*u*). Water overflows around the rim of the vessel into the funnel (*v*), and is carried away through the outer tube (*w*). The funnel is supported on the box (*c*). The surface may be observed through the microscope (*x*), and the camera lucida attachment (*y*) used to measure the angle of contact. The details of the mounting of the protractor wheel (*e*) on the axis (*j*) of the specimen holder, and the suspension of the platform (*z*) from the rails (*h*), are also shown.

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